# Characterization of lymph node stromal cells during Treg-mediated tolerance

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

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Basel, 2013

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät

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# Abbreviations

| | |
|---|---|
| Ab | antibody |
| ABM | anti-bm12 specific T cells |
| Allo | allogeneic |
| APC | antigen presenting cell |
| ATG | anti-thymocyte globulin |
| Bax | Bcl-2-like protein 4 |
| Bcl-2 | B-cell lymphoma 2 |
| Bcl-xL | B-cell lymphoma-extra large |
| BEC | blood endothelial cells |
| CCL | chemokine ligand |
| CCR | chemokine receptor |
| CD | cluster of differentation |
| cDNA | complementary deoxyribonucleic acid |
| DAMPs | danger associated molecular patterns |
| DCs | dendritic cells |
| dLNs | draining lymph nodes |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DNCs | double negative cells |
| DP | double positive |
| ELISA | enzyme-linked immunosorbent assay |
| FACS | fluorescence activated cell sorting |
| FBS | fetal bovine serum |
| FITC | fluorescein isothiocyanate |
| Foxp3 | forkhead box protein 3 |
| GATA3 | GATA- binding protein 3 |
| GFP | green fluorescent protein |
| GM-CSF | granulocyte-macrophage colony stimulating factor |
| GVHD | graft versus host disease |
| HBSS | Hanks' Balanced Salt Solution |
| HEV | high endothelial venules |
| HSCT | hematopoietic stem cells transplantation |
| i.p. | intraperitoneal |
| i.v. | intravenous |
| ICAM | intercellular cell adhesion molecule |
| IDO | indolamin-2,3-dioxygenase |
| IFN- | interferon |
| IFN-γ | interferon gamma |
| IL | interleukin |

| | |
|---|---|
| ITGA | integrin alpha chain |
| iTreg | inducible regulatory T cells |
| JAK | Janus kinase |
| KO | knock out |
| LAG3 | Lymphocyte-activation gene 3 |
| LEC | lymphatic endothelial cells |
| LFA-1 | lymphocyte function-associated antigen-1 |
| LN | lymph node |
| LNSC | lymph node stromal cells |
| MFI | mean fluorescence intensity |
| MHC | major histocompatibility complex |
| min | minute(s) |
| mRNA | messenger ribonucleic acid |
| NO | nitric oxide |
| NOD | nucleotide oligomerization domain |
| NOS | nitric oxide synthase |
| nTreg | natural regulatory T cells |
| PAMPs | pathogen associated molecular patterns |
| PD-1 | programmed cell death 1 |
| PD-L1 | programmed cell death ligand 1 |
| PE | phycoerythrin |
| PI | propidium iodide |
| pMHC | peptide-MHC |
| PRRs | pattern recognition receptors |
| PTA | peripheral tissue antigens |
| RORγ | RAR-related orphan receptor gamma |
| RT | room temperature |
| RT-PCR | real time polimeraze chain reaction |
| $S1P_1$ | sphingosine 1-phosphate receptor-1 |
| SD | standard deviation |
| SEM | standard error mean |
| SLO | secondary lymphoid organ |
| SMA | smooth muscle actin |
| STAT | signal transducer and activator of transcription |
| Syn | syngeneic |
| TCR | T cell receptor |
| TGF | Tumor growth factor |
| Th | T helper |
| TIM 3 | T cell immunoglobulin and mucin domain-3 protein |
| TNF | tumor necrosis factor |
| TRC | T zone reticular cells |
| Treg | regulatory T cells |

| | |
|---|---|
| TSLP | thymic stromal lymphopoietin |
| TSLPR | thymic stromal lymphopoietin receptor |
| VCAM | vascular cell adhesion molecule |
| WT | wild type |

# Summary

Solid organs transplantation is therapy of choice for several human diseases. The success of this treatment is determined by the recipient's ability to prevent rejection. Rejection is currently controlled by immunosuppressive drugs, which unfortunately have side effects for patient quality of life as well as long-term graft survival. It is therefore important to better understand the molecular mechanisms underlying tolerance and rejection.

Regulatory T (Treg) cells are known to play an important role in suppressing immune responses, and have been demonstrated to be important for establishing allograft tolerance. To study Treg-mediated suppression we have generated a skin transplantation model in which the transfer of polyclonal Treg cells suppresses anti-Bm12 specific CD4 T cells (ABM cells) in a ratio dependent manner. Firstly, we characterized the migration pattern of both ABM and Treg cells to the skin graft and to the dLNs. High numbers of Treg cells delay the proliferation and reduce the IFN-γ production of ABM cells in the LNs. The release of IFN-γ by activated T cells was shown to influence the LN microenvironment comprised of stromal cells, which provide a structural environment for the homeostasis and differentiation of lymphocytes. Furthermore, T zone reticular cells (TRCs) were shown to provide survival factors and negatively regulate activated T cells during strong inflammatory responses. We showed that the presence of Treg cells was modifying the TRC activity during the establishment of tolerance. TRCs isolated from LNs of mice tolerating the graft exhibited enhanced transcription of the chemokines CCL19 and CCL21, the adhesion molecules ICAM-1 and VCAM-1 and the cytokines TSLP and IL-7 compared to TRCs isolated from mice transferred with ABM cells only. In our

analysis we focused on the survival factor IL-7 and its role in activation. We found that IL-7 signaling in activated Treg cells leads to further upregulation of the IL-2Rα-chain CD25 and enhances Treg suppressive capacity in vitro.

In summary, Treg cells suppress activation and IFN-γ release by effector T cells and promote TRC release of IL-7. We hypothesize that TRC derived IL-7 may be required to maintain high numbers of Treg cells in the LNs and to prevent skin graft alloresponses. In addition, IL-7 might sensitize Treg cells and facilitate their recognition of IL-2, leading to Treg cell expansion and better suppression of effector T cell responses.

# 1 Introduction

## 1.1 The immune system

The immune system protects the host against damaging inflammatory responses, which occur for example during infection, tumor and autoimmunity. The detection of inflammatory responses results in the engagement of innate and adaptive immune responses, which act together to efficiently eradicate pathogens and injured cells. While innate immunity is rapid and non specific, adaptive immunity is antigen specific and long lasting (1) (2).

### 1.1.1 Innate immunity

Innate immunity is highly conserved among different species, underscoring its importance for host survival. Innate immune cells, including both professional antigen-presenting cells (APCs) (i.e. dendritic cells, monocytes, macrophages and B cells) and non-professional antigen presenting cells (i.e. endothelial and epithelial cells), express pattern recognition receptors (PRRs). PRRs recognize pathogen and danger associated molecular patterns (PAMPs and DAMPs), which induce signal transduction, secretion of cytokines, chemokines and other metabolites. The release of these molecules promotes the recruitment of immune cells required to resolve the inflammation. While all cells can present antigens on major histocompatibility complex (MHC) class I molecules, only APCs can engulf and present immunogenic antigens on MHC class II molecules. Maturation and activation of innate immune cells are strongly influenced by the recognition via different PRRs, including Toll-like receptors (TLRs) and nucleotide oligomerization domain

(NOD) receptor family. The maturation of host cells contributes to the activation of adaptive immune cells, promotes and mediates cytotoxic response and the development of long lasting immunity. In addition to innate cells, the complement system has been shown to have an important role during early immune responses. It consists of serum glycoproteins that become activated in a cascade sequence with amplification stages.

### 1.1.2 Adaptive immunity

The successful induction of a primary adaptive immune response requires presentation of antigenic peptides on MHC molecules to activate naïve T lymphocytes. The majority of T cells express a specific T-cell receptor that allows them to recognize cognate antigenic peptides displayed by APCs in secondary lymphoid organs (SLO). Antigens are processed in cellular compartments and, depending on their localization, are presented on MHC class I or II molecules. Cytosolic antigens are presented on MHC class I to activate T cells expressing the CD8 co-receptor. Antigens in the degradative compartment, including phagosomes and lysosomes, are presented on MHC class II to activate T cells expressing the CD4 co-receptor. Activated CD8 T cells have cytolytic activity while CD4 T cells support other cells in targeting and eliminating foreign antigens. $CD4^+$ T cells, also called T helper cells, can be further subdivided into Th1, Th2, Th17 and induced Treg (iTreg) cell subsets. Once activated, adaptive immune cells migrate to the inflamed tissue and start an effector response by cell-cell contact and cytokine release. A peculiarity of the adaptive immune response is the formation of immunological memory, which allows a fast reactivation of antigen-specific T cells after a second exposure to the same antigenic peptide.

#### 1.1.2.1 T cell selection in the thymus

To ensure an efficient defense of the host without recognition of self-antigens, lymphocytes must be selected during development. Here I will focus on central tolerance and T cells selection (3, 4).

Hematopoietic stem cells in the bone marrow give rise to early thymic precursors that migrate to the thymus and develop into mature T cells. The thymus is subdivided into two regions; the outer cortex, which contains immature thymocytes and the inner medulla, which contains mature T cells. Early thymic precursors are double negative for the co-receptors CD4 and CD8 and express an unselected αβ TCR repertoire consisting of a pre-Tα-chain and a β-chain. The expression of the αβ TCR drives the clonal expansion and differentiation of $CD4^-CD8^-$ thymocytes into $CD4^+CD8^+$ double positive (DP) thymocytes, which undergo different selection processes.

In the thymus, 90% of the DP thymocytes express αβ TCRs that fail to recognize the peptide-MHC (pMHC) ligand and are therefore eliminated by a process called “death by neglect”. The remaining 10% DP thymocytes are either positively selected into the mature T cell compartment or eliminated by negative selection. Positive selection occurs when thymocytes recognize low affinity antigen in the context of self-MHC while negative selection occurs due to strong TCR interactions with pMHC. Negative selection results in the elimination of self-reactive T cells and ensures that the peripheral T cell pool is largely self-tolerant. Nevertheless, it is clear that some autoreactive T cells escape negative selection and enter the periphery where they undergo deletion or anergy induction upon encounter with self-antigen. In addition, a more recently identified population of $CD4^+CD25^+$ regulatory T cells (Tregs) has been demonstrated to exert dominant control over the activation of autoreactive T cells.

## 1.2 CD4+ T cells

### 1.2.1 T helper cells

$CD4^+$ T cells arise in the thymus from single positive $CD4^+CD8^-$ precursors. Once they migrate to the periphery they recognize antigens presented on MHC class II molecules expressed by APCs. Depending on the activation state of the APCs and the cytokine environment present at the time of antigen encounter, $CD4^+$ T cells can differentiate into four major subsets: Th1, Th2, Th17 and iTreg cells (5-8) (Fig. I).

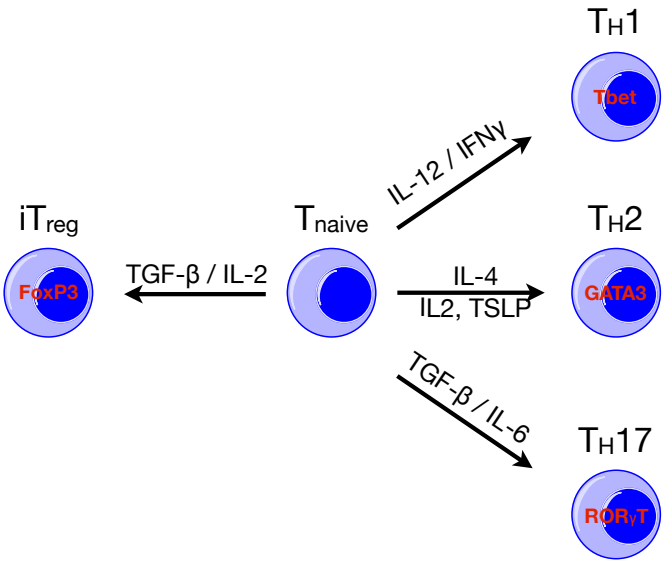



**Figure I. Differentiation of T helper cell subsets** (as reviewed in (8, 9)). Particular cytokine environments differentiate naïve CD4 T cells into different subsets (Th1, Th2, Th17, iTreg) that have distinct function in the regulation of immune responses. Each subset is characterized by a specific transcription factor (T-bet, GATA3, ROR-γt and FoxP3).

Th1 cells develop in response to IL-12, produced by macrophages and APCs upon encounter with several microbial products, and IFN-γ, produced by Th1 cells in a positive feedback loop (5, 8, 9). They are characterized by the expression of the transcription

factor T-bet and the signal transducer and activator of transcription (STAT) molecule STAT4. Th1 cells secrete IL-2, IFN-γ and tumor necrosis factor-beta (TNF-β) and activate macrophages to kill engulfed pathogens and recruit other immune cells to the site of infection. Indeed, Th1 cells are responsible for cell-mediated immunity against intracellular pathogens. However, these cells need to be strongly controlled since they have been also implicated in the immunopathology of certain organ-specific autoimmune diseases (10-13). In addition, unwanted Th1 responses against donor-antigens are an obstacle to successful graft tolerance following organ transplantation (14).

Th2 differentiation is driven mainly by IL-4 (15) and TSLP (16-18) and is characterized by the expression of GATA3 and activated STAT5, which promote gene transcription of IL-4, IL-5, IL-9, IL-13 and IL-25 cytokine genes. Th2 responses regulate the elimination of extracellular pathogens and antibody-mediated immunity, by attracting and activating eosinophils and basophils, and by promoting the synthesis of IgE antibodies (9).

Th17 responses differentiate in the presence of TGF-β and IL-6 and are characterized by the ROR-γt transcription factor and STAT3 activation. Through the production of IL-17 and IL-22, Th17 cells control immune responses against extracellular bacteria and fungi.

iTreg cells differentiate in the presence of TGF-β and IL-2 and express factor forkhead box P3 (FoxP3) and STAT5. iTreg cells maintain peripheral immune tolerance and regulate immune responses (9).

Importantly, the cytokine production of each subset negatively regulates the other to ensure only one polarization of the immune response under specific conditions (5).

#### 1.2.1.1 T cell migration in vivo

The continuous migration of T cells within the body is regulated through various adhesion and activation events that occur between T cells and the vascular epithelium. T cells from

the blood enter the LN via specialized high endothelial venules (HEVs). In the LN, naïve and memory T cells scan for antigens before returning to the circulation via the lymphatics. In contrast to steady state conditions, inflammatory signals and activated DCs increase the recruitment to the LN by inducing chemokines and adhesion molecules (19). The interactions between adhesion molecules of the vascular endothelium and the activated T cells signal to induce firm arrest which facilitates the entry into the LN and other tissues (20). Naïve $CD4^+$ T cells express high levels of the L-selectin adhesion molecule (CD62L) that binds to the glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1) expressed on HEVs of the LNs (21). This interaction is fundamental for T cell homing to SLOs since it allows the firm adhesion and rolling of free-floating T cells on HEVs. Subsequently, the expression of the chemokine receptor 7 (CCR7) on T cells is important for the transmigration after binding with chemokine-ligand 19 (CCL19) and chemokine-ligand 21 (CCL21) expressed on HEVs and within the LNs (22). CCR7 signaling activates integrins such as lymphocyte function-associated antigen-1 (LFA-1) and very late antigen-4 (VLA-4) on T cells to facilitate binding on intercellular adhesion molecule-1 and 2 (ICAM-1/2) and vascular cell adhesion molecule-1 (VCAM-1) respectively to transmigrate through the HEVs (23). All of these adhesion molecules are not only involved in cell arrest for proper migration but also have an important role in the formation and stabilization of immunological synapses during T cell priming (24).

T cells egress from the LN and other SLOs depends on the expression of the sphingosine-1-phosphate receptor 1 (S1P1) in response to high concentration of the S1P ligand present in the lymph and blood (25). Once back in the circulation, other chemokine receptors guide activated T cells to inflamed tissues. In particular the chemokine receptor CCR4 is required to drive the migration of T cells to inflamed skin (26).

#### 1.2.1.2 Costimulatory molecules influencing TCR signals

T cell activation leads to the up-regulation and down regulation of various surface molecules involved in effector function and recognition of microenvironmental signals. CD69 is a surface glycoprotein (27) known as early leukocyte activation molecule and acts as a costimulatory molecule for T-cell activation and proliferation (28). In addition, CD69 was described to inhibit the function of $S1P_1$ (29), and to have an immunomodulatory role in down regulating immune responses through the production of TGF-β (30). CD44 is a cell-surface glycoprotein that binds to extracellular matrix component via the glycosaminoglycan hyaluronic acid (HA). Effector and memory T cells upregulate CD44 (31), which in turn modulates growth, survival, differentiation and motility (32). CD44 has also been shown to regulate the recruitment of inflammatory T cells to the skin by binding to E-selectin on the skin endothelium (33, 34). While naïve T cells express high levels of the IL-7 receptor (IL-7R) (35), activated T cells express the IL-2 receptor (α-chain) CD25 (36) (see Chapter 1.2.1.3).

In addition, inhibitory molecule including programmed cell death 1 (PD-1) and T cell immunoglobulin and mucin domain-3 protein (TIM-3) are expressed on fully activated effector Th1 cells and define the phenotype of exhausted effector T cells (37-39). PD-1 interacts with two possible ligands: PD-L1, which is expressed on leukocytes and non-hematopoietic cells, and PD-L2 that is expressed exclusively on dendritic cells and monocytes (40-42). Engagement of PD-1 by either of its ligands during TCR signaling block T-cell proliferation, cytokine production, cytolytic function and impair T-cell survival by preventing the induction of the survival factor Bcl-xL and the expression of T cells subsets-specific transcription factors (37, 43). In particular, PD-1/PD-L1 interaction was shown to promote tolerance by increasing T cells mobility and preventing stable

conjugates between T cells and APCs (42). TIM-3 is expressed on a variety of cells including activated DCs, Treg cells and Th1 cells. Once an effector Th1-cell response is generated, terminally differentiated Th1 cells express TIM-3, which then upregulate the expression of the TIM-3 ligand, galectin-9, through the production of IFN-γ. Finally, galectin-9 binds TIM-3 on the surface of Th1 cells and inhibits their response by triggering cell death (38, 44). Blocking the TIM-3 pathway results in hyperproliferation of Th1 cells with massive IFN-γ production and abrogates allotolerance (39, 45). Indeed, both PD-1 and TIM-3 molecules activate signaling pathways which are important for the termination of immune responses.

#### 1.2.1.3 Survival and proliferation signaling in T cells

Common cytokine receptor γ-chain ($\gamma_c$) family cytokines have crucial roles in the development, proliferation, survival and differentiation of different cell linages. The receptors for those cytokines share the $\gamma_c$ that form dimers or trimers with cytokine-specific chains (46). Here I will focus on IL-7 and IL-2 signaling.

IL-7 is produced mainly by stromal cells, epithelial cells and fibroblasts and has a central role in the development of T cells in both human and mice (47, 48). Abrogation of IL-7 signaling leads to severe defects in T cell development and lymphopaenia (49). In addition, IL-7 was shown to be involved in LN organogenesis (50) and is recognized as a potent lymphocyte survival factor in the periphery (51, 52). IL-7 signals through the IL-7R, which consist of a dimer formed between the $\gamma_c$ and the IL-7R α-chain (Fig. II). IL-7 signaling is important for the induction of B-cell lymphoma 2 (Bcl-2) molecule, which prevents apoptosis in T cells (53, 54). Indeed, Bcl-2 overexpression rescued IL-7R-deficient T cells (55, 56) and partially but not completely restored T cell numbers and function. These data suggest an additional role for IL-7 and IL-7R signaling beyond Bcl-2

induction. Indeed, IL-7 was shown to play a key role in preventing atrophy and maintaining resting T cells metabolism (57).

Interestingly, the IL-7R α-chain is shared by an IL-7-like cytokine, thymic stromal lymphopoietin (TSLP) although it does not belong to the $\gamma_c$ family (58). Indeed, the TSLP receptor consists in the IL-7R α-chain in combination with the TSLPR which is closely related to the $\gamma_c$ chain (Fig. II). TSLP was described to be preferentially expressed by epithelial cells within different tissues, including lung, skin and gut (58). In the periphery, TSLP is mainly described to be involved in the generation of Th2 responses by modulating DCs (16-18, 58). In the thymus, the release of TSLP by human thymic epithelial cells activates a subpopulation of DC to express CD80 and CD86, which may be critical for the differentiation of auto-reactive T cells into Treg cells (59).

In addition to the survival factor IL-7, IL-2 is an important cytokine produced by activated T cells which is required for T cells growth and proliferation. Moreover, IL-2 is essential for Treg cells expansion and mice deficient in IL-2 signaling have severe autoimmune disorders (60). IL-2 signals via the trimeric IL-2R, which consist of the $\gamma_c$ together with IL-2R α- and IL-2R β-chains (Fig. II). Both IL-7R and IL-2R activate signal transduction pathways that involve the tyrosine kinases Janus kinase 1 (JAK1) and JAK3 and mainly the STAT molecule STAT5 (46). TSLPR in combination with IL-7Rα activates Jak1, Jak2 and STAT5. Inhibitory molecules like PD-1 can inhibit STAT5 phosphorylation and therefore negatively affect T cells proliferation and survival (61).

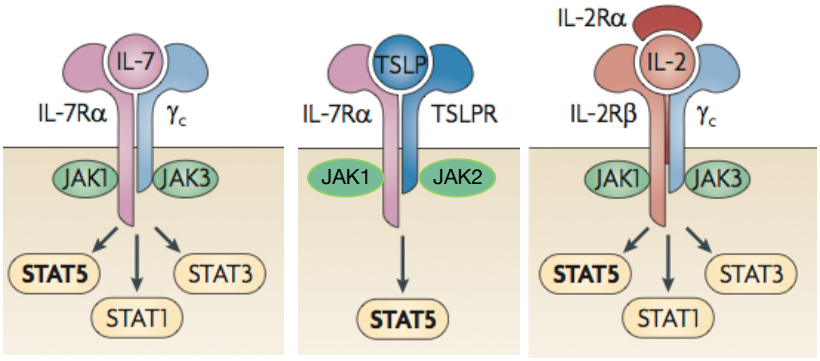



**Figure II. IL-7, TSLP and IL-2 receptors and signal transduction** (adapted from (46)). Dimeric IL-7 and TSLP receptor and the trimeric IL-2 receptor are represented. The major signal transduction molecules are represented and the main signal transducer and activator of transcription (STAT) protein STAT5 is shown in bold.

## 1.2.2 Regulatory T cells

### 1.2.2.1 Definition, phenotype

To function properly, the immune system must discriminate between self and non-self; when this discrimination fails, the immune system destroys cells and tissues of the body and as a result causes autoimmune disease. Regulatory T (Treg) cells are a specialized subpopulation of CD4 T cells that suppress the activation of the immune system, thereby maintaining immune homeostasis and tolerance to self-antigens (62).

Treg cells are traditionally defined as $CD4^+$ T cells that constitutively express the α-chain of the IL-2 receptor, CD25. Furthermore, they constitutively express the transcription factor forkhead box P3 (FoxP3) which can be used for their identification and isolation and is required for their development (63) and suppressor function (64). Mice that carry a spontaneous loss-of-function mutation or deletion of the FoxP3 gene, known as scurfy

mice, develop an autoimmune-like disease with hyper-responsive $CD4^+$ T cells. These data underscore the importance of Treg cells in the maintenance of peripheral tolerance (65).

Treg cells can arise via two distinct developmental pathways. Naturally occurring Treg cells (nTreg) are selected in the thymus. They represent 5% of mature $CD4^+CD8^-$ thymocytes (66) and 5-10% of the $CD4^+$ T cells in the periphery (67). Since they are selected by autoantigens, they express a high affinity autoreactive TCR repertoire (67) that allows to suppress immune responses to self antigens in the periphery and thereby prevent autoimmunity (68, 69). Alternatively, $CD4^+$ T cells that are activated by antigens in the periphery in a tolerogenic microenvironment can develop into induced Treg cells (iTreg) that also express high levels of the transcription factor FoxP3 (70). Interestingly, FoxP3 expression on iTreg was shown to be unstable compared to the expression on nTreg, which is probably related to the different methylation of the FoxP3 locus in the two Treg populations (71). The conversion from effector T cells to iTreg cells requires TCR and CD28 signaling and specific cytokines, like TGF-β and IL-2 (68). The microenvironment, therefore, plays a fundamental role in the generation of iTreg cells from effector cells in order to prevent destructive immune responses.

#### 1.2.2.2 Suppressive mechanism

The molecular mechanism by which Treg cells exert their suppressor/regulatory activity has not been definitively characterized and is the subject of intense investigation. Four main mechanisms have been proposed (Figure III).

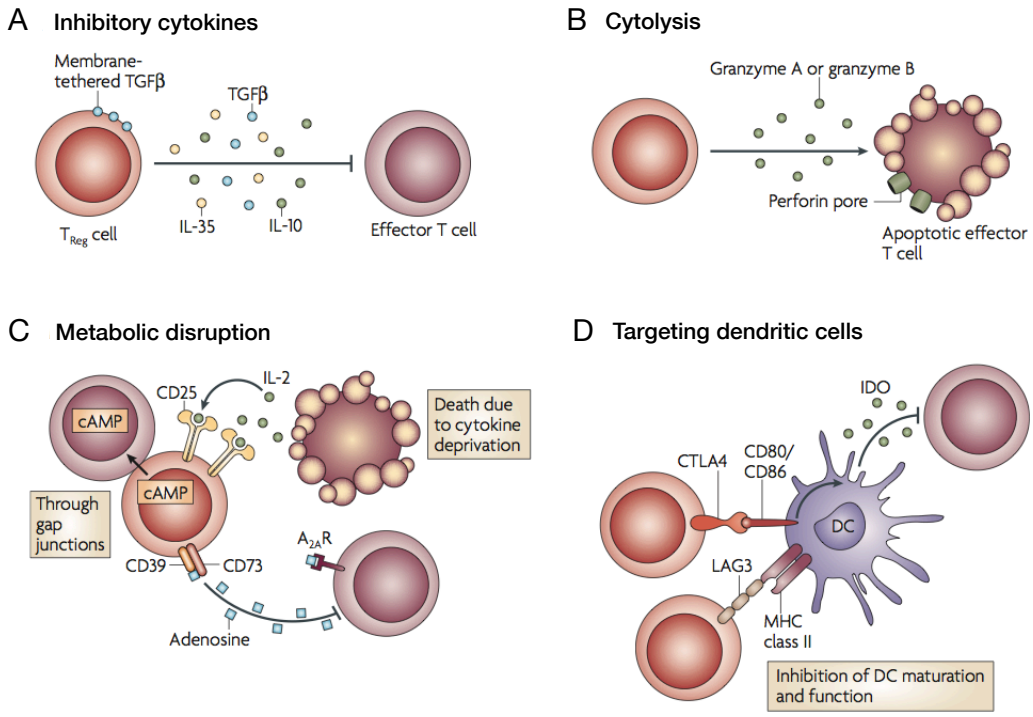



**Figure III. Treg cells suppression mechanisms** (72). (A) Inhibitory cytokine suppression via IL-10, IL-35 and TGF-β. (B) Cytolysis of target cells via granzyme A or B and perforine. (C) Metabolic disruption of IL-2 via high expression of CD25 and of adenosine via expression of CD39 and CD73. Cyclic AMP (cAMP) transfer in the target cells through gap junction inhibit IL-2 production. (D) Suppression by targeting and impairing maturation and function of DCs.

Treg cell can produce inhibitory cytokines including IL-10, IL-35 and transforming growth factor β (TGF-β) that directly act on effector T cells to suppress a wide spectrum of cellular activities (Figure III, A). IL-10 strongly inhibits the establishment of Th1 responses and the activation of APCs leading them to a tolerogenic phenotype. IL-35 and TGF-β are strong inhibitors of T cell proliferation. In addition TGF-β is involved in the maintenance of nTreg and the induction of iTreg creating a tolerogenic environment (73).

Treg cells have been shown to induce cytolysis of NK and CD8 T cells in tumors in a granzyme B- and perforin dependent fashion upon activation (Figure III, B). Interestingly,

the cytotoxic activity of Treg cells was induced by the tumor environment and the deficiency of granzyme B resulted in successful clearance of tumor cells (74).

Treg cells can exert metabolic disruption as another suppressive mechanisms (Figure III, C). As mentioned before, Treg cells constitutively express the α-chain of the IL-2 receptor, CD25 that allows them to consume the IL-2 in the surrounding environment. Since Treg cells are unable to produce IL-2, they must compete for IL-2 produced by activated T cells and in doing so, actively starve dividing cells and deprive them from this essential survival factor (75, 76). In addition, it was shown that the pericellular accumulation of adenosine elicits immunosuppressive cellular responses (77). The extracellular adenosine is generated by two important ectoenzymes that are expressed on the surface of Treg cells, CD39 and CD73. CD39 hydrolyzes adenosine tri- and diphosphate (ATP and ADP) to adenosine monophosphate (AMP). Extracellular AMP is, in turn, rapidly degraded to adenosine by CD73. The accumulation of pericellular adenosine can elicit immunosuppressive cellular responses by the interactions with the adenosine receptors ($A_{2A}R$) expressed on activated T cells (77). Moreover, Treg cells harbor high levels of cyclic AMP (cAMP) that via gap junctions can be directly transferred into effector T cells leading to inhibition of proliferation and IL-2 synthesis (78).

Treg cells were shown to suppress the maturation and function of DCs (Figure III, D). Cytotoxic T-lymphocyte antigen 4 (CTLA-4) interacts with co-stimulatory molecules CD80 and CD86 on DCs that impair the priming of effector T cells and induce indoleamine 2,3-dioxygenase (IDO) production. IDO is known to be a potent regulator of effector functions by inducing the production of pro-apoptotic metabolites from the catabolism of tryptophan (79). On the other hand, lymphocyte-activation gene 3 (LAG3) expressed on Treg cells binds with high affinity the MHCII molecule on DCs and, by activating inhibitory

signaling pathways, suppresses DC maturation and their immunostimulatory capacity (80).

#### 1.2.2.3 Regulation of homeostasis and suppressive capacity of Treg cells

While IL-2 was shown to be the main factor involved in intrathymic Treg cells development, IL-7 mediates Treg cell development in the absence of IL-2 (81-83).

Similarly, IL-2 was shown to be important for peripheral homeostasis (60, 84) and suppressor function of Treg cells (76). Indeed, mice deficient for IL-2 or IL-2R showed a decreased number of Treg cells in the thymus and periphery leading to autoimmune diseases (60). Naïve $CD4^+$ T cells and memory T cells have been shown to rely on IL-7 for their peripheral homeostasis (35, 85). However the role of IL-7 in the homeostasis of Treg cells is still unknown. In the last years, several studies reported that Treg cells express IL-7R (86-88) and require IL-7 for their homeostatic proliferation (86, 88). Whether IL-7 is important for Treg cells function is still not known.

In addition to environmental factors, cell-cell contact molecules might influence Treg cells activity. The PD-L1 expression on non-hematopoietic and hematopoietic cells in the presence of TGF-β promotes the *de novo* generation of Treg cells (37). The negative costimulation receptor PD-1 was shown to be expressed on Treg cells and it was recently reported that Treg cells from PD-1 KO mice were unable to suppress CD8 T cell responses against tumors (89). Interestingly, Treg cells also express PD-L1, which seems to have important implications in the maintenance of high Treg numbers and possibly the generation of additional iTreg cells (37).

Recently a new $PD1^+$ Treg population was identified by coexpression of TIM-3 in skin graft at the time of rejection (90). $PD1^+$ Treg cells exhibited higher in vitro effector function and an increased suppressive capacity following skin transplantation. However, $TIM\text{-}3^+$

Treg cells did not proliferate when transferred into newly transplanted host providing evidence that they are a short-lived Treg cell population.

## 1.3 The lymph node

Lymph nodes (LNs) are SLO with a highly organized structure and are located at the points where vessels of the lymphatic system converge. The LN consists of a capsule containing an outer cortex and an inner medulla. The cortex can be subdivided into different areas. The outer part consists of B cells organized in primary or secondary lymphoid follicles that contain the germinal centers where B cells proliferation occurs during an immune response. The inner paracortical area contains mostly T cells and dendritic cells. Macrophages and plasma cells are present in the medulla and make up the inner medullary cords (1). A dense network of reticular fibers maintains this compartmentalized structure with functionally different microenvironments. Follicular dendritic cells (FDCs) (91, 92) are present in the B cell zone and produce CXCL13 to attract and retain B cells, while CCL19 and CCL21 produced by T zone reticular cells (TRC) exert the same function for T cells and antigen presenting cells (APCs) (Figure IV). Lymph draining from extracellular spaces of the body enters through the afferent lymphatic vessels located beneath the capsule (subcapsular sinus) and exit the LN via the efferent lymphatics in the medulla. Lymphocytes recirculating in the blood are able to directly enter the LN through specialized blood vessels known as high endothelial venules (HEVs) (93). These specialized post-capillary venules are composed by plump endothelial cells which constitutively express adhesion molecules and chemokines at their luminal surface thereby allowing the continuous transmigration of lymphocytes (94).

Similar to other SLO, LNs fulfill three major functions in the body. First, they filter extracellular fluids from the tissues to sample antigens, PAMPs and DAMPs present in the body. Second, they are the organs where adaptive immune responses are initiated. APCs from infected tissues migrate to the LN where they encounter and prime naïve immune

cells coming from primary lymphoid organs, thymus and bone marrow. Third, LNs provide a structural environment for the homeostasis and differentiation of lymphocytes.

### 1.3.1 Lymph node stromal cells

#### 1.3.1.1 Localization, phenotype and characterization

Lymph node stromal cells (LNSC) are non-hematopoietic mesenchymal cells with fibroblast properties that form the architectural scaffold of the LN. By creating a specialized conduit system, LNSC facilitate and ensure an efficient T cells encounter and crosstalk with APCs (95-99). Recently, four different LNSC subsets have been identified by the expression of the adhesion molecule CD31 (PECAM-1) and the glycoprotein podoplanin (gp38). T zone reticular cells (TRC, also known as follicular reticular cells (FRC)) are $gp38^{+}CD31^{-}$. Lymphatic endothelial cells (LEC) are $gp38^{+}CD31^{+}$. Blood endothelial cells (BEC) are $gp38^{-}CD31^{+}$, and a fourth population is double negative (DNC) for both markers (97) (Figure IV).

LECs and BECs surround lymphatic and blood vessels respectively and regulate the migration of immune cells into and out of lymph nodes by the expression of chemokines and adhesion molecules. LECs have recently been described to be involved in peripheral tolerance by direct presentation of peripheral tissue antigens (PTA) to CD8 T cells. As a result, autoreactive CD8 T cell proliferation is inhibited and CD8 T cells can be deleted due to both a lack of costimulation and active PD-L1 engagement (100). Less is known about the function of the DNC subset. Although DNCs closely resemble TRCs in their gene expression profile they have recently been described to contain pericyte-like cells with highly contractile function (101). These cells express the highest level of α-smooth muscle actin (α-SMA) transcripts and over half of them are positive for integrin $\alpha_7$

(ITGA7). For this reason, they have been newly termed "ITGA7$^{+}$ pericytes" (IAPs). Recent data indicate that DNCs surround some of the medullary and cortical vessels (101) and are suggested to be important for the expansion and contraction of vessels during immune responses (confidential data from A. Fletcher, AAI Conference 2012). TRCs are the major LNSCs and secrete extracellular matrix components to form a dense reticular network and the lymph draining conduit system (95, 102). Furthermore, they have been shown to both positively and negatively influence immune responses (101, 103-105).

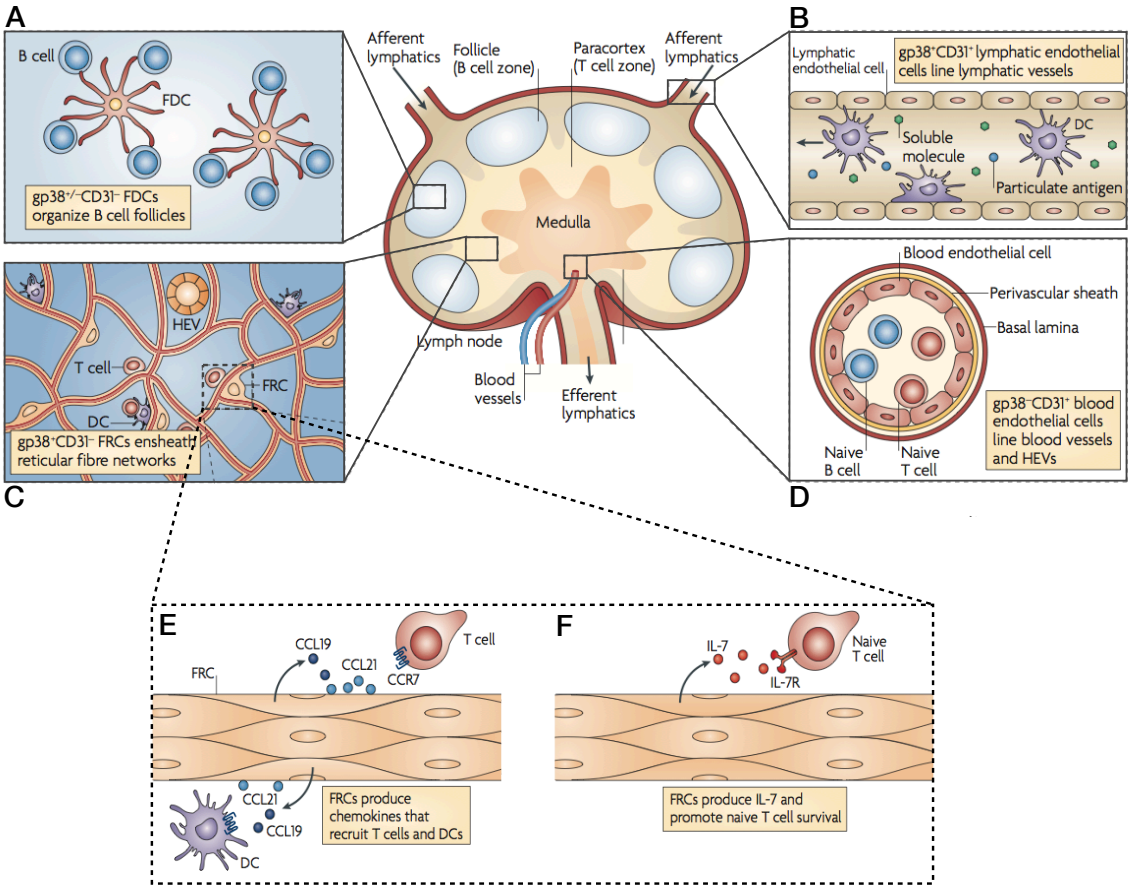



**Figure IV. Location of lymph node stromal cells in the LN** (adapted from (106)). (A) Follicular dendritic cells (FDCs) are located in B cells follicles. (B) Lymphatic endothelial cells (LECs) surround lymphatic vessels. (C) T zone reticular cells (TRCs) networks are found in T

cell zones in the paracortex of the lymph node where T cells and DCs encounter each other. (D) Blood endothelial cells (BECs) form specialized blood vessels known as high endothelial venules (HEVs) which are surrounded by a perivascular sheath and a basal lamina. (E) TRCs produce the chemokines CCL19 and CCL21 to attract and guide T cells and DCs along the three-dimensional network. (F) TRCs produce the cytokine IL-7 to promote naïve T cells survival.

#### 1.3.1.2 TRCs as positive regulators of T cell responses

TRCs have been shown to positively regulate T cells responses by various mechanisms. Once T cells enter the LN via the HEVs they immediately associate with and crawl along the TRC network which functions as a local road to define the path of T cell migration within the LN parenchyma. TRCs might facilitate T cells in their search for cognate antigens presented by DCs colocalized to the same TRC network. In addition, since TRCs are almost exclusively present in the paracortex and naive T cell movement is largely restricted to the TRC network, the latter also defines the T cell zone in the LN and is likely to play a key role in preventing naive T cell entry into B cell follicles (95, 96). Both the migration and the confinement in these regions of the LN are dependent on the secretion of specific chemokines. Indeed, TRCs are the main constitutive producers in the LN of the homeostatic cytokines CCL19 and CCL21 that are recognized by CCR7 molecules expressed on the surface of incoming naïve T cells and dendritic cells (Figure IV, E). The lower average motility and velocity of adoptively transferred CCR7-deficient $CD4^+$ T lymphocytes observed in T cell zones of wild type (wt) recipients (107) confirmed the central role of CCR7 receptor signaling in enhancing these processes (108, 109). Moreover, CCL19 has been shown to play an important role not only in the motility but also in the survival of naïve T cells (97) as well as in the maturation of dendrite formation and antigen uptake by dendritic cells (105, 110, 111).

TRCs are the main local source of IL-7 in the LN (97) (Figure IV, F). IL-7 is a stromal-cell derived cytokine that has important function during LN organogenesis (50). In addition, IL-7 regulates lymphocyte development in bone marrow and thymus as well as naïve and memory T cells homeostasis in the periphery (51) by modulating T cell fitness and survival. Indeed, during HIV-1 infection, loss of the FRC network and access to IL-7 leads to the apoptosis of naïve T lymphocytes (112). In addition, IL-7 seems to have an important role in enhancing TCR signaling and primary antigen-specific T cell expansion (113, 114). Within the LN, IL-7 does not diffuse freely but is immobilized by extracellular matrix components (115). This allows the local provision of highly concentrated IL-7 to cells trafficking through the LN and defines a constrained niche for naïve cells and memory cells (115, 116). In addition, IL-7 was shown to be involved in LN remodeling following viral infection in both humans and mice (117).

#### 1.3.1.3 TRCs as negative regulators of T cell responses

Many recent studies have reported the negative impact of TRCs on immune responses. Similar to BEC and LEC, TRCs have been shown to express peripheral tissue antigens (pTA) (103, 118-121), suggesting an important role for LNSC in the establishment of CD4 and CD8 peripheral T cell tolerance. The expression of inducible nitric oxide synthase (iNOS) depends on nitric oxide (NO) production by TRCs and is a key molecule in down regulating T cells expansion and tolerance induction (118, 121). Besides their role in promoting tolerance, TRCs are also important for dampening acute T cell immune responses (104, 120, 122). Although TRCs do not affect initial T cell priming, they exert control over the survival and proliferation of activated T cells (105). NO has a central role also in this process and is released by TRCs after synergistic IFN-γ and TNFα stimulation (104, 120). Activated CD8 T cells produce these cytokines in the LN less then 24h after T

cell priming. NO is a free radical that is rapidly captured by neighboring cells and negatively regulate T cells by different mechanism. In human it was shown to down regulate the TCR complex by nitrosylating diverse amino acid residues (123). In addition, the consumption of the iNOS substrate L-arginine down regulates the ζ chain of the TCR and inhibits T cell proliferation (124). NO was shown to block the phosphorylation of the "signal transduction and transducer 5" (STAT5) thereby interfering with IL-2 signaling and production by activated T cells (125).

Another IFN-γ-dependent suppressive mechanism is mediated by programmed cell death ligand 1 (PD-L1). PD-L1 is up-regulated on TRCs as well as on LECs (126) during acute immune responses (104, 120). The interaction of PD-L1 with the receptor PD-1 expressed by activated T cells blocks early activation signals promoted by CD28 and up-regulation of the high affinity IL-2 receptor (37, 126). Moreover, PD-1 engagement prevents the induction of the cell survival factor Bcl-xL as well as expression of transcription factors associated with T cell effector function and appears to promote the development and function of Treg cells (37).

IFN-γ was shown to be the major regulator of FRC activity. Thus, IFN-γ exhibits both positive and negative influences on immune responses. On the one hand, IFN-γ acts as a pro-inflammatory cytokine that promotes macrophage activation, clearance of intracellular pathogens, up-regulation of MHC molecules and promotion of T-helper 1 (Th1) responses. On the other hand, IFN-γ also limits tissue disruption by interrupting immune responses that are too strong (105).

## 1.4 Transplantation

Organ transplantation is the surgical operation by which a failing or damaged organ in the recipient is substituted with a healthy organ from the donor. In 1954 Joseph Murray performed the first successful kidney transplantation on identical twins (127). Nowadays transplantation is an important medical intervention and can be performed for different organs, and tissues. There are different transplantation types: autografts are transplants within the same individual; syngrafts and allografts are transplants from genetically identical and genetically unrelated individuals respectively.

### 1.4.1 MHC restriction

The discovery of MHC restriction shed light on the phenomenon of rejection of MHC mismatched organs and tissues (1). Positive T cell selection in the thymus and bone marrow restricts T cell recognition of MHC. Following migration to the periphery, T cells recognize peptides presented only by self-MHC molecules. However, when T cells encounter peptide presented by non-self MHC (i.e. MHC present in the transplanted organ), they undergo an alloresponse characterized by strong proliferation and effector differentiation. Even when donor and recipient are completely MHC matched, an alloresponse may occur due to minor histocompatibility antigens, protein from the grafted tissues that are presented to T cells. Allogeneic T cells are the main players in transplant rejection.

### 1.4.2 Direct and indirect allorecognition

A critical event in the rejection of a grafted organ is the presentation of donor alloantigens by DCs that migrate from the graft to the draining LNs to initiate T-cell priming and

differentiation into effector cells. Alloantigens can be presented to the recipient T cells in two ways (128). In the first phase, resident donor DCs migrate from the graft to draining LN (dLNs) where they activate specific T cells that recognize intact donor MHC molecules. In the second phase, recipient DCs process and internalize donor MHC antigens in the graft and then migrate to dLNs to present donor Ag on self-MHC molecules to recipient T cells (128).

### 1.4.3 Different types of rejection

Donor organs can be rejected early or late after transplantation (1). Hyperacute rejection is caused by preexisting alloantibodies present in the recipient at the time of transplantation. This process can happen within minutes. An acute rejection occurs within weeks after engraftment and is driven by activation of allogeneic T cells. However, engraftment of organs does not lead to life long acceptance and transplanted organs can still be rejected in a process called chronic rejection. The major cause for chronic graft rejections is progressive atherosclerosis, fibrosis and athrophy of blood vessels, which normally supply the grafted organ. Furthermore, infections and activation of immune responses might change the microenvironment and break established tolerance.

### 1.4.4 Transplantation treatments in the clinics

To avoid rejection of the transplanted organ, patients must take lifelong immunosuppressive drugs, which can have serious side effects. It is therefore of primary interest to find new immunological targets to reduce immunosuppression and improve the quality of life of transplanted patients.

One of the main strategies used to promote allograft acceptance is the administration of lymphodepleting agents. Paradoxically, depletion of lymphocytes may predispose the

recipient to reject the transplanted organ by promoting the development and expansion of alloreactive memory T cells (116). Indeed, following depletion, remaining adaptive lymphocytes undergo homeostatic proliferation in order to repopulate the peripheral lymphoid compartment. During this process T cells acquire a memory-like phenotype and the ability to respond to low doses of antigen, even in the absence of costimulation. These cells would therefore become more dangerous because more resistant to depletion and costimulation blocking treatments (116, 129). The combination of depleting approaches with therapies that sustain the development and expansion of Treg cells would be the best strategy in treating allograft rejection.

The fate of an alloimmune response is dependent on the balance between alloreactive effector T cells and Treg cells in vivo (130).

In recent years thymoglobulin, a rabbit polyclonal anti-thymocyte globulin (ATG) widely used for induction therapy in clinical transplantation, was shown to prolong graft survival by depleting peripheral T cells and preferentially sparing natural Treg cells (131). This treatment thereby limits the generation of dangerous alloreactive cells arising after homeostatic proliferation (132).

Furthermore, many transplant recipients are nowadays treated with a monoclonal antibody specific for CD25, the IL-2 receptor α chain. However both activated effector T cells and Treg cells express high levels of CD25 making them susceptible to this treatment. For this reason the impact of the anti-CD25 antibody administration on the generation and expansion of Treg cells is still under debate (70).

Rapamycin is a well known immunosuppressive drug that targets the mammalian target of rapamycin (mTOR) pathway that has been shown to sustain both Treg cell generation ex vivo (133, 134) and function in vivo (135).

### 1.4.5 Clinical Treg cells therapy

In addition to immunosuppressive therapies that promote Treg cells induction in vivo, patients may also be treated with an infusion of Treg cells shortly before transplantation or during an acute rejection episode (136). Ex vivo expanded thymic-derived Treg cells were shown to clinically improve two patients that developed graft versus host disease (GVHD) after bone marrow transplantation (137). An alternative treatment for patients with chronic GVHD is low-dose IL-2 therapy (138) which was shown to increase the median number of Treg cells.

There are two main concerns with the use of Treg therapies in the clinics. The transfer of high numbers of Treg cells may lead to a global immunosuppression. Despite this concern, patients infused with Treg cells were not reported to be more susceptible to infection (139). Secondly, the heterogeneity of the human FoxP3+ population and the absence of exclusive marker for human Treg cells make their discrimination from other T cells difficult. Indeed, human Treg cells are so far identified as $CD25^{high}$ and $CD127^{low}$, a phenotype shared by activated T cells. Interestingly, it has been shown (133) that effector T cells are particularly sensitive to the anti-proliferative effects of rapamycin. For this reason, the addition of rapamycin to Treg cells cultured under expanding conditions selectively increases the purity of Treg cells.

However, these therapies were mainly described in GVHD and hematopoietic stem cells transplantation (HSCT) patients and whether or not they can also be used for solid organ transplantation remains to be determined.

### 1.4.6 Skin transplantation

Skin transplantation is used in a variety of clinical situations, including skin reconstitution after major burn injuries (140). However, skin grafts are rejected in an acute fashion and immunosuppressive agents known to be effective in preventing organ transplant rejection, have little or no effect on skin transplantation. For these reasons, clinical skin transplantation is currently confined to autografts (141). It is therefore of primary importance to study the rejection process in mouse models in order to understand and target new mechanisms for the establishment of skin transplantation tolerance.

# 2 Material and Methods

## 2.1 Mice

Wild-type inbred C57BL/6/J, Rag2$^{-/-}$/J, B6.129S7-IFN-gr1tm1Agt/J (Jackson Laboratory), IFN-γ$^{-/-}$ (142), CD3$^{-/-}$ (143), B6.C-H-2Bm12 (144), TCR transgenic ABM Rag2$^{-/-}$ mice reactive to MHCII I-A$^{bm12}$ (145), FOXP3eGFP (146), and Bm12 Rag2$^{-/-}$ as well as Bm12 Ly5.1 (generated in this study) were bred in the Animal House of the Department of Biomedicine, University Hospital Basel and Füllingsdorf, Switzerland according to the regulations of Swiss veterinary law.

## 2.2 Antibodies and reagents

Monoclonal antibodies recognizing TCR Vα2 (B20.1), TCR Vβ8.1, 8.2 (KJ16-133.18), CD3 (145-2C11), CD4 (GK1.5), CD11c (N418), CD16/32 (93), CD25 (PC61), CD31 (390), CD40 (3/23), CD44 (IM7), CD45.2 (104), CD62L (MEL-14), CD69 (H1.2F3), CD80 (16-10A1), CD86 (GL-1), CD140a (APA5), CD197 (4B12), CD274 (10F.9G2), CD279 (RMP1-30), podoplanin (gp38, 8.1.1), IFN-γ (XMG1.2), FOXP3 (150D), MHC-II (AF6-120.1, 25-9-17), fluorochrome-labeled streptavidins, and the fixation and permeabilization kit with BD GolgiStop were purchased from Biolegend and BD Pharmingen. Specificity of staining was confirmed with isotype-matched control antibodies. The proliferation dye eFluor670 was purchased from eBioscience. Recombinant IL-7, GM-CSF and IFN-γ (Prepro Tech). IL-7 complex was formed by incubation of recombinant mouse IL-7 and M25-Ab (kindly provided by Daniela Finke, DBM, Basel) to stimulate IL-7R signaling on various cell types.

Sandwich ELISAs were purchased from BD Biosciences and R&D Systems. Phorbol 12-myristate 13-acetate and ionomycin were purchased from Sigma, Collagenase IV from Worthington, DNaseI and Collagenase D from Roche.

## 2.3 Preparation of lymph node cells

Naïve transgenic Vα2Vβ8 $CD4^+$ T cells were isolated from lymph nodes of ABM mice. Treg cells were sorted from FOXP3eGFP mice. Lymph node stromal cell were isolated according to the protocol adapted from Link et al., 2007 (97). Briefly lymph nodes were opened with needles and first digested in DMEM (1.2 mM $CaCl_2$, 2% FCS, Pen/StreP) containing collagenase IV (1mg/ml) and DNase1 (40 mg/ml) to isolate lymphocytes. In a second digestion step with DMEM containing collagenase D (1mg/ml) and DNase1 (40 mg/ml), stromal lymph node cells were isolated and used for experiments or further sorted to isolate subpopulations.

## 2.4 Sorting

GFP positive Treg cells were sorted with a 70-µm tip at a pressure of 60 psi. Dendritic cells and lymph node stromal cells were sorted by CD45, CD11c, gp38 and CD31 staining with a 100-µm tip at a pressure of 30psi. The purity of sorted cells routinely exceeded 98% with an Influx cell sorter (BD).

## 2.5 Flow cytometry

Cell suspensions were stained in ice-cold HBSS supplemented with 2% (vol/vol) FCS for surface staining. For intracellular cytokine staining, cells were stained for surface markers, were fixed and then resuspended in permeabilization buffer (BD) containing anti-IFN-γ,

anti-FoxP3eGFP. Data were acquired on a FACS Canto II (BD Biosciences) and analyzed with FlowJo software (TreeStar). If required, PI staining to exclude dead cells was performed directly before flow cytometry analysis.

## 2.6 Skin transplantation model

Donor mice were transplanted on the back with full-thickness tail-skin from allogeneic Bm12, or syngeneic mice under general anesthesia with Isofluorane. A piece of ca. 1cm$^2$ of back skin of the recipient mouse was removed and substituted with a piece of tail skin of the same size from the donor. Grafts were glued using Histoacryl® (Aesculap AG, Tuttlingen, Germany) and covered with Vaseline lubricated finger strips (Hansaplast, Beiersdorf, Germany). After 7 days, bandages were removed and mice were kept for an additional 14 days to allow wound healing. After complete wound healing, naïve ABM and Treg cells were adoptively transferred (i.v.) into recipient mice at different ratios. Graft appearance was assessed until rejection and for a maximum of 100 days using the following scoring system: 3, short hair, black stripe, shiny greyish appearance; 2, small red area, loss of hair shine and dryness; 1, large red area, no black stripe and dryness; and 0, rejected. At indicated time points, lymph nodes and skin grafts were collected and cells were isolated.

## 2.7 RT-PCR from cells

mRNA was isolated from either single cell suspension of T cells, DCs and stromal cells or total lymph node cells by μMACS™ mRNA Isolation Kit. Reverse transcription was carried out using One-step cDNA Kit from Miltenyi Biotech according to the manufacturer's protocol. Amplification was performed in 10μl with GoTaq qPCR Master Mix (Promega)

using Real-Time PCR (Applied Biosystems 7900HT). The following program was used: 40 cycles of 50°C for 2 min, 95°C for 2 min, 95°C for 15 s, 60°C for 1 min. Primers for RT-PCR were designed using Universal ProbeLibrary Assay Design Center from Roche Applied Biosystems and synthesized by Microsynth (Balgach, Switzerland) and are listed in Supplements.

## 2.8 Generation of bone-marrow derived DCs

DCs were generated as described (147). Briefly, bone marrow cells from tibia and femur were flushed with RPMI medium. $CD11b^+$ BMDCs (DCs) were generated by plating bone marrow progenitors in complete RPMI 1640 (with Pen/Strep, 2-mercaptoethanol (all from Invitrogen), 10% (vol/vol) heat-inactivated FBS (BioConcept) and supplemented with 10% conditioned medium from GM-CSF transduced X63 (148). After 3 days, non-adherent cells were removed and attached cells were further cultured in supplemented RPMI medium. On day 5 to 8, cells were harvested and analyzed for $CD11c^+$ expression which was routinely 80-90% positive.

## 2.9 Expansion of FRC like stromal cells ex vivo

After digestion, stromal cells were plated in six-well plates at a density of $20 \times 10^6$ cells per well in complete RPMI medium. After 24h and 48h, non-adherent cells were removed and adherent cells were further incubated for 5 days. Adherent cells were detached with 0.05% Trypsin (GIBCO) and stromal cells were isolated by CD45 depletion (Miltenyi Biotech). Purified stromal cells were 12% CD31+ and 84% gp38+ and contained less than 2% CD45+ cells.

## 2.10 Stimulation of stromal and dendritic cells

For analysis of cytokine production and surface marker expression, $5x10^4$ LNSCs with/without $2x10^5$ DC per well were cultured in 96 well plates in 200 µl RPMI 1640 containing the indicated cytokines. Surface expression of selected proteins was analyzed by flow cytometry and cytokines were quantified by sandwich ELISA according to the manufacturer's instructions (BD Biosciences).

## 2.11 In vitro T cell stimulation assay

$5 \times 10^3$ LN cells were seeded in 96-well plates for 4h. EFluor670 stained ABM cells ($5 \times 10^3$/well) and different ratio of Treg cells ($5 \times 10^3$/well to $5 \times 10^4$/well) were incubated in RPMI medium containing 8U/ml hrIL-2 and $2 \times 10^4$ CD3/CD28 dynabeads (Invitrogen). In some experiments recombinant cytokines or cytokines antibody complexes were added. At indicated time points supernatants were collected to determine cytokine release and cells were analyzed for mRNA expression, proliferation, and surface markers expression by flow cytometry.

## 2.12 MACS separation

Cells were separated using positive and negative cell isolation kits (Miltenyi) for isolation of $CD11c^+$ DCs and $CD45^-$ stromal cells with AutoMACS according to manufacturer's instructions.

## 2.13 Assessment of protein expression

Total proteins were extracted in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1% sodium deoxycholate, 0.1% SDS, and phosphatase/protease inhibitors (Roche). Protein concentration was determined using BCA protein assay kit (Pierce Biotechnology). Five µg of proteins were resolved on 10% SDS-PAGE and transferred on PVDF membranes. After 2h blocking in 5% milk TBST (TBS, 0.2% Tween 20), the membranes were incubated with primary antibodies diluted in 1% milk TBST (over night, 4°C) followed by washing steps and incubation with horseradish peroxidase - conjugated secondary antibody (2h, room temperature). Proteins were visualized using ECL Luminata Forte/Crescendo Western HRP Substrate (Millipore) and exposed to X-ray film (Fuji Medical X-Ray Film). Revelation was performed in an Agfa Processor and bands densitometry was analyzed with ImageJ Software

## 2.14 Statistical analysis

Statistical analyses were performed using Prism (GraphPad Software, Inc.). Data for expression of mRNA, cytokines and surface protein expression were analyzed using the one-way ANOVA with Bonferroni's multiple comparison test or the Mann-Whitney t test or unpaired two tailed student's t test. Graft survival was analyzed using the Kaplan-Meier Log-rank (Mantel Cox) test. P values $< 0.05$ are considered as statistically different.

## 2.15 Primer List

| Primer Name | Sequence (5' -> 3') | Primer size | Amplicon size |
|---|---|---|---|
| β-actin f | ACG TAG CCA TCC AGG CTG TG | 20 | 124 |
| β-actin r | TGG CGT GAG GGA GAG CAT | 18 | |
| CCL19 f | TGT GGC CTG CCT CAG ATT AT | 20 | 122 |
| CCL19 r | AGT CGG CCG CAT CAT TAG CAC | 21 | |
| CCL21 f | TCC AAG GGC TGC AAG AGA | 18 | 92 |
| CCL21 r | TGA AGT TCG TGG GGG ATC T | 19 | |
| IAb f | GTG GTG CTG ATG GTG CTG | 18 | 76 |
| IAb r | CCA TGA ACT GGT ACA CGA AAT G | 22 | |
| ICAM-1 f | CCC ACG CTA CCT CTG CTC | 18 | 72 |
| ICAM-1 r | GAT GGA TAC CTG AGC ATC ACC | 21 | |
| IFN-γR1 f | TCA AAA GAG TTC CTT ATG TGC CTA | 24 | 93 |
| IFN-γR1 r | TAC GAG GAC GGA GAG CTG TT | 20 | |
| IL-2R f | TGT GCT CAC AAT GGA GTA TAA GG | 23 | 72 |
| IL-2R r | CTC AGG AGG AGG ATG CTG AT | 20 | |
| IL-7 f | AAA GCC AGA GCG CCT GGG TG | 20 | 108 |
| IL-7 r | CTG GGC AGG GCA GTT CAG GC | 20 | |
| Nos2 f | GGG CTG TCA CGG AGA TCA | 18 | 66 |
| Nos2 r | CCA TGA TGG TCA CAT TCT GC | 20 | |
| Nos3 f | CCA GTG CCC TGC TTC ATC | 18 | 66 |
| Nos3 r | GCAGGGCAAGTTAGGATCAG | 20 | |
| PD-1 f | TGC AGT TGA GCT GGC AAT | 18 | 77 |
| PD-1 r | GGC TGG GTA GAA GGT GAG G | 19 | |
| PD-L1 f | CCA TCC TGT TGT TCC TCA TTG | 21 | 76 |
| PD-L1 r | TCC ACA TCT AGC ATT CTC ACT TG | 23 | |
| TSLP f | GGA CTG TGA GAG CAA GCC AGC TT | 23 | 91 |
| TSLP r | TGT TTT GTC GGG GAG TGA AGG GC | 23 | |
| VCAM-1 f | TGG TGA AAT GGA ATC TGA ACC | 21 | 86 |
| VCAM-1 r | CCC AGA TGG TGG TTT CCT T | 19 | |

# 3 Aim of thesis

Treg cells are known to play an important role in suppressing immune responses, and have been demonstrated to be important for establishing allograft tolerance. Suppression of immune responses can be either established in the allograft or in the LNs. The LN is composed of stromal cells that form a conduit system and provide a structural environment for homeostasis and differentiation of lymphocytes. Moreover, LNSCs were shown to provide survival factors and negatively regulate activated T cells during strong inflammatory responses. However, the role of LNSCs during alloresponses and Treg-mediated tolerance is still unknown.

The aim of my doctoral thesis was to establish a skin transplantation model in which the transfer of polyclonal Treg cells suppresses anti-bm12 specific effector CD4 T cells capable of mediating graft rejection and to characterize the microenvironment of the LN at different time points. Of particular interest was the interaction of Treg cells and TRCs during establishment and maintenance of skin allograft tolerance in mice.

# 4 Results

## 4.1 Establishment of skin transplantation model in Rag2$^{-/-}$ mice

To study the role of Treg-mediated tolerance and its consequences for the LN microenvironment, we chose the bm12 skin transplantation model. A spontaneous three point mutation in the antigen presenting groove of the IA$^{b}$ molecule (IA$^{bm12}$) was shown to elicit an alloimmune response and graft rejection in C57BL/6 mice (144). Consistent with published data (144), C57BL/6 WT recipient mice rejected the graft within 14 days (Fig. 1). To confirm the dependence of graft rejection on T cells, T-cells deficient CD3e$^{-/-}$ and T- and B-cells deficient Rag2$^{-/-}$ mice were also transplanted. Both, CD3e$^{-/-}$ and Rag2$^{-/-}$ mice showed prolonged graft survival up to 100 days (Fig. 1).

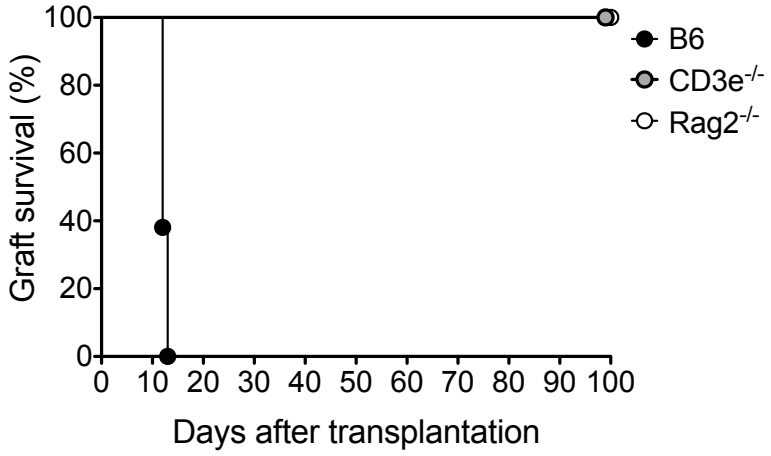



**Figure 1. CD3e$^{-/-}$ and Rag2$^{-/-}$ mice are tolerant for the Bm12 skin graft.** C57BL/6 WT (black, n = 21), CD3e$^{-/-}$ (gray, n = 6) or Rag2$^{-/-}$ (white, n = 15) recipient mice received bm12 skin allografts on day 0. Graft survival is displayed as Kaplan-Meier plot. The difference

between C57BL/6 and the other recipient groups is statistically significant ($p < 0.0001$) using Log-rank (Mantel Cox) test.

However, adoptive transfer of Treg cells into bm12-transplanted C57BL/6 mice did not prevent skin graft rejection and depletion of C57BL/6 mice with anti-CD3 was not sufficient to induce graft tolerance (personal communication, Simona Rossi).

Bill et al. identified that monoclonal anti-bm12 (ABM) specific $CD4^+$ effector T cells express the Vα2Vβ8 TCR and acquire effector functions after encounter with the cognate antigen (149)**.** Adoptive transfer of 2 x $10^4$ transgenic ABM cells in $Rag2^{-/-}$ mice induced graft rejection within 12 days, which was delayed by adoptive transfer of polyclonal Treg cells (Fig. 2). Co-injection of 2-fold more Treg cells prolonged graft survival up to 16 days and injection of 10-fold more Treg cells (2 x $10^5$) induced graft tolerance up to 100 days (Fig. 2).

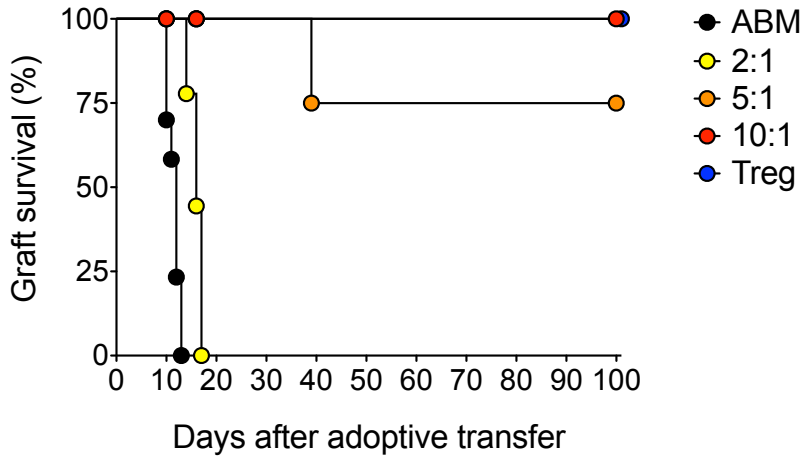


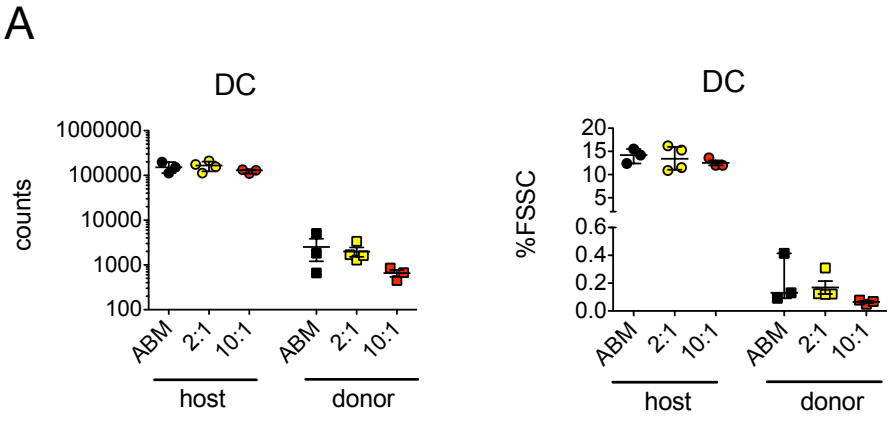

**Figure 2. Treg cells induce prolonged Bm12 skin graft survival.** Allograft transplanted $Rag2^{-/-}$ mice ($n \geq 7$) were adoptively transferred with ABM cells (black) or Treg cells (blue) alone or in combination at different Treg : ABM ratios (yellow, orange, red) on day 0. Graft

survival is displayed as Kaplan-Meier plot. The difference between the groups is statistically significant ($p < 0.0001$) using Log-rank (Mantel Cox) test.

## 4.2 Migration and homing of ABM and Treg cells to skin grafts and LNs

To elicit an immune response, T cells have to encounter DCs in the LN displaying the cognate antigen. Treg mediated suppression in the LN might change the recruitment and maturation of DCs. To address this possibility, we analyzed host DCs (Fig. 3, circles) residing in the LNs of transplanted mice 6 days after T cell adoptive transfer. DC number and frequency as well as activation marker expression including MHC class II (IAb), CD40, CD80 and CD86 were similar in the ABM, 2:1 and 10:1 group (Fig. 3, B).

Next we investigated the migration of DCs from donor Ly5.1 bm12 grafts on day 0 (data not shown) and day 6 after adoptive T cell transfer (Fig. 3, squares). Donor DCs were detectable in similar numbers and frequencies in the draining LN of transplanted mice. Their expression of MHC class II and co-stimulatory molecules CD40, CD80 and CD86 was higher compared to the expression on host DCs, albeit not different in the ABM, 2:1 and 10:1 group (Fig. 3, B). These results suggest that the presence of Treg cells does not impair the migration and activation of skin graft-derived donor DCs.



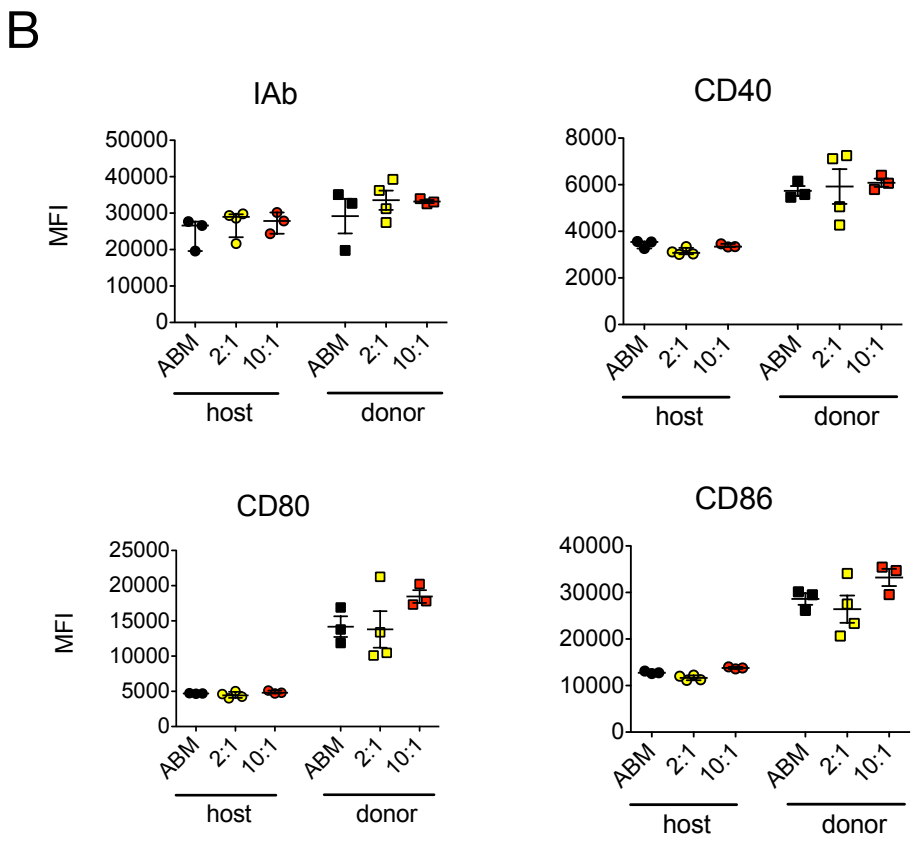



**Figure 3. Treg cells do not affect numbers and phenotype of host and donor DCs in LNs.** (A) Ly5.1$^{-}$ CD11c$^{+}$ host DCs (circles) and Ly5.1$^{+}$ CD11c$^{+}$ donor DCs (squares) numbers (left) and frequencies (right) and (B) their phenotype was determined using flow cytometry for IAb, CD40, CD80 and CD86. ABM alone (black), and the Treg:ABM ratios 2:1 (yellow) and 10:1 (red) group 6 after adoptive T cell transfer. n ≥ 3. Data are representative of one experiment out of two. Differences between the groups were not significant using the Mann Whitney t test. Error bars denote interquartile range.

Next we investigated the recruitment of ABM and Treg cells to the skin graft and their expression of the skin homing receptor, CCR4, and the activation marker CD25. Recruitment of both T cell subsets was similar in all groups until day 9 (Fig. 4, A). The expression of CCR4 on ABM cells transferred at a 2:1 ratio with Treg cells was higher compared with ABM cell transferred alone, while in the 10:1 group ABM express CCR4 in

a comparable manner to the ABM group. However the different expression of CCR4 did not correlate with higher ABM counts in the skin graft of the 2:1 group. Treg cells had similar levels of CCR4 expression in the 2:1, 10:1 and Treg group. The expression of CD25 on ABM cells was comparable in all groups, while Treg cells expressed higher levels of CD25 in the 10:1 group compared to Treg transferred alone (Fig. 4, B).

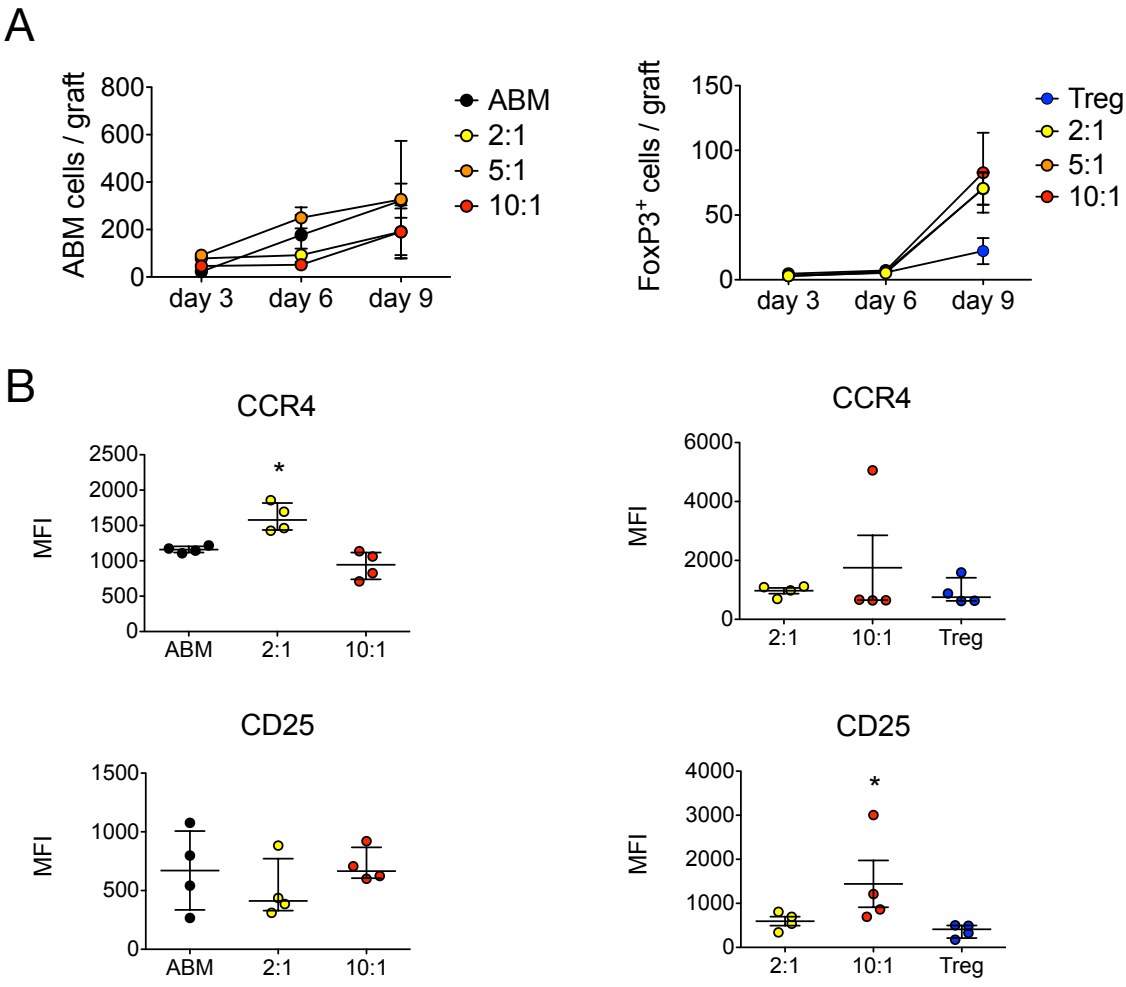



**Figure 4. Migration and phenotype of ABM and Treg cells following Bm12 skin graft transplantation.** (A) ABM cells (left) were detected via Vα2Vβ8 TCR staining and Treg cells (right) via FoxP3eGFP expression in the skin graft in the ABM (black), 2:1 (yellow), 5:1 (orange), 10:1 (red) and Treg (blue) group by flow cytometry on day 3, 6 and 9 after adoptive T cells transfer. Cell number per skin graft is plotted over the time after adoptive T cells

transfer. Error bars denote SEM. n ≥ 4. (B) Surface expression of CCR4 and CD25 on ABM cells (left) and Treg cells (right) was determined by flow cytometry on day 9. Statistical analysis was performed using the Mann Whitney t test by comparing to the ABM or Treg group. *, $P < 0.05$. Error bars denote interquartile range. n = 4.

The presence of Treg cells in the 2:1 and 10:1 group might create a tolerogeneic milieu in the skin graft within 9 days. To test this we transplanted C57BL/6 mice with skin grafts removed from mice transferred with 2:1 and 10:1 Treg:ABM cell ratios and compared them with grafts removed from mice receiving ABM cells alone. All grafts were rejected by day 35, suggesting that Treg cells establish tolerance in the LN and not in the graft (Fig. 5).

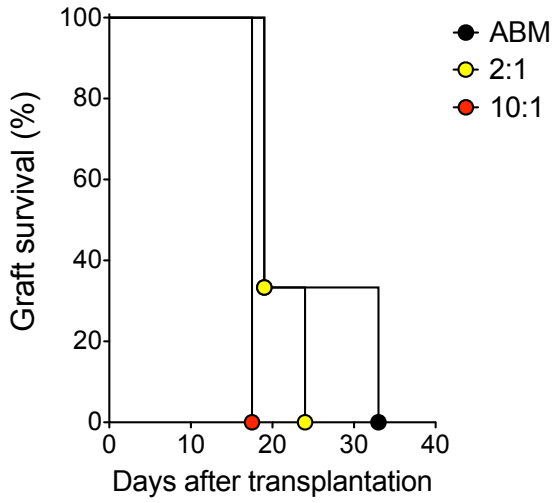



**Figure 5. Treg cells do not establish tolerance in skin grafts.** C57BL/6 WT recipients were transplanted with Bm12 allografts removed from the different group (ABM (black, n = 3), 2:1 (yellow, n = 3) and 10:1 (red, n = 2)) of $Rag2^{-/-}$ transplanted mice 9 days after adoptive T cells transfer. Graft survival is displayed as Kaplan-Meier plot. The difference between the groups is not statistically significant.

All groups showed comparable migration of ABM cells to a pool of axillary, brachial and inguinal LNs on days 6 and 9 (Fig. 6, A). On day 9, the number of ABM cells was higher in

the absence of Treg cells and significantly lower in the 2:1, 5:1 and 10:1 group (Fig. 6, A and B). However, Treg cells were present in comparable numbers in the LNs on day 6 and 9 (Fig. 6, A and B). Interestingly, by calculating the ratio of Treg cell to ABM cell numbers at 9 days after adoptive transfer, we found that a ratio >1 correlates with long term tolerance of the skin graft (Fig. 6, C). Taken together, our data indicate that ABM mediated skin graft rejection can be suppressed by double amount of Treg cells in the LN.

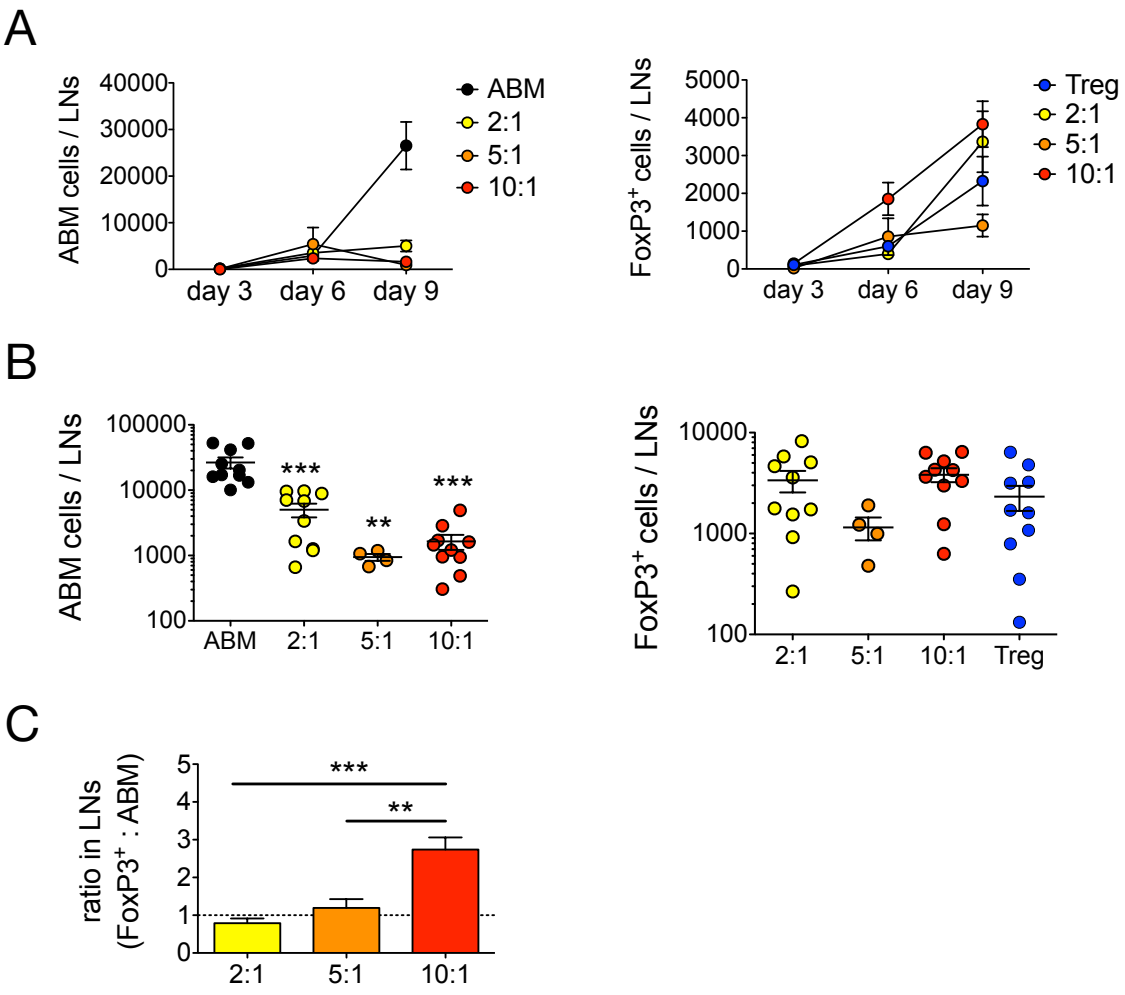



**Figure 6. Treg cells reduce number of ABM cells in the LNs of transplanted mice.** (A) Counts of Vα2$^+$Vβ8$^+$ ABM cells (left) and FoxP3eGFP$^+$ Treg cells (right) were quantified in

the LNs in the ABM (black), 2:1 (yellow), 5:1 (orange) and 10:1 (red) group by flow cytometry on day 3, 6 and 9 after adoptive T cells transfer. (B) Counts of ABM (left) and Treg cells (right) on day 9 were compared with 2:1, 5:1 and 10:1 groups using the Mann-Whitney t test. **, $P < 0.01$; ***, $P < 0.001$. Error bars denote SEM. (C) Treg : ABM cells ratio in the 2:1, 5:1 and 10:1 group in the LN on day 9. The dotted line represents the threshold (ratio = 1). Statistical analysis was performed using a One-way analysis of variance with Bonferroni's Multiple Comparison test (significant for P value $< 0.05$). $n \geq 4$. Error bars denote SEM.

## 4.3 Bm12 mediated ABM and Treg cells expansion in LNs

Under lymphopenic conditions, both homeostatic proliferation and survival modulate T cell expansion in the LNs. To understand the relative contributions of homeostatic proliferation and Ag-driven proliferation, we compared allografts and syngrafts from $Rag2^{-/-}$ mice on day 9. In allografted mice, decreased numbers of ABM cells were recovered in the presence of transferred Treg cells (Fig. 6, B). In syngrafted mice, ABM cell numbers were independent of the number of Treg cells (Fig. 7, A, left), indicating that Treg cells suppress only Ag-driven proliferation of ABM cells. Next we analyzed the percentages of recovered ABM cells in the LNs with the formula (no. $ABM_{day\ 9}$ / no. $ABM_{day\ 0}$)*100. In LNs of allotransplanted mice, ~94%, ~25% and ~7% of injected ABM were recovered in the ABM, 2:1, and 10:1 group respectively. In syngrafted mice, only 1% of ABM cells were recovered in all groups (Fig. 7, A, right).

In allografted mice, Treg cell numbers were similar in all groups (Fig. 6, B) and in syngrafted mice Treg cell numbers were higher in the 10:1 and Treg group (Fig. 7, B, left). Interestingly, in allografted mice ~7% of Treg cells were recovered in 2:1 group, ~2% in the 10:1 group and ~1% in the Treg group. In syngrafted mice only ~1% of Treg cells were detected in the LNs in all groups (Fig. 7, B, right). These data suggest that ~1% of

naïve ABM and Treg cells survive in the LNs and that bm12-activation of ABM cells might lead to expansion of both ABM and Treg cells.

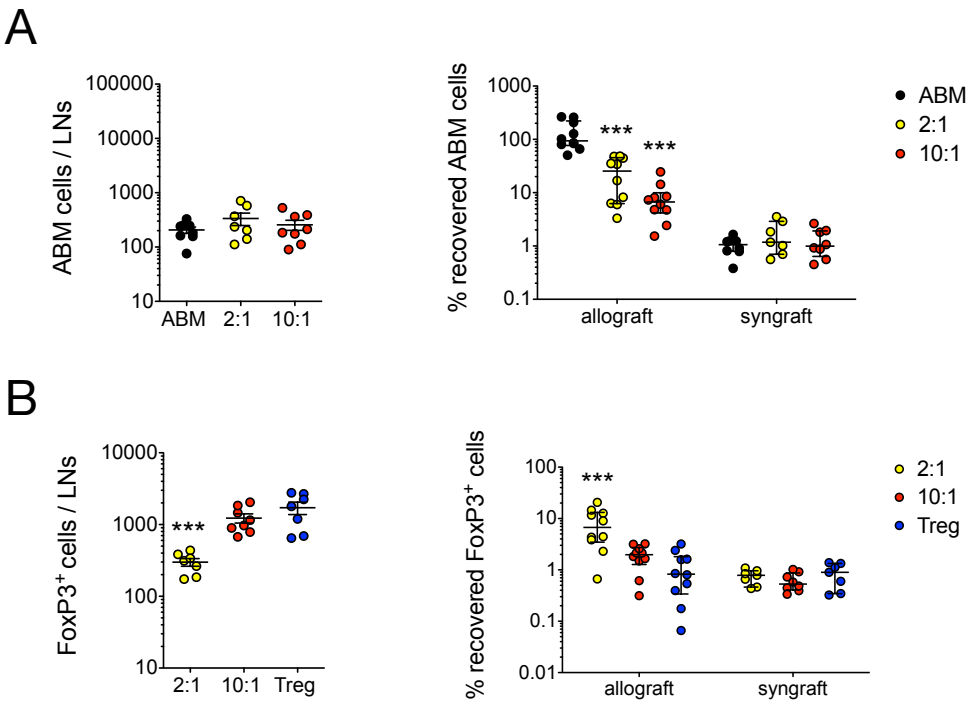



**Figure 7. Bm12-specific ABM and Treg cells expansion in LNs.** (A) Counts of $V\alpha2^+V\beta8^+$ ABM cells in the LNs of syngrafted mice (left). Percentage of recovered ABM cells ((no. $ABM_{day\ 9}$ / no. $ABM_{day\ 0}$)*100) (right) in allograft and syngraft of transplanted mice in the different groups. (B) Counts of Foxp3eGFP Treg cells in the syngrafted mice (left). Percentage of recovered Treg cells (right) in allograft and syngraft of transplanted mice in the different groups. Statistical analysis was performed using the Mann Whitney t test by comparing to the control (ABM or Treg) group. *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$. Error bars denote SEM. n = 7

## 4.4 Phenotypical analysis of ABM cells in the presence of Treg cells

We next analyzed the activation status of ABM cells in the skin draining LNs of transplanted mice 6 (data not shown) and 9 days (Fig. 8, A) after adoptive transfer of T cells (ABM, 2:1, 10:1 groups).

Six and nine days after adoptive transfer, the surface expression of the LN-homing chemokine receptor CCR7 was similar in all groups. CD62L and IL-7R were downregulated after adoptive transfer (data not shown), but this downregulation was independent of the presence of Treg cells. The early activation marker CD69 was expressed more highly in the 10:1 group than in the ABM group on day 9. CD44 and CD25 as well as the inhibitory TIM-3 and PD-1 molecules were comparable in all groups (Fig. 8). With the exception of CD44 and the upregulation of CD69 in the 10:1 group, expression of these surface markers was similar in syngrafted mice (data not shown), suggesting that the activation of ABM cells is induced by homeostatic proliferation and is not inhibited by Treg cells.

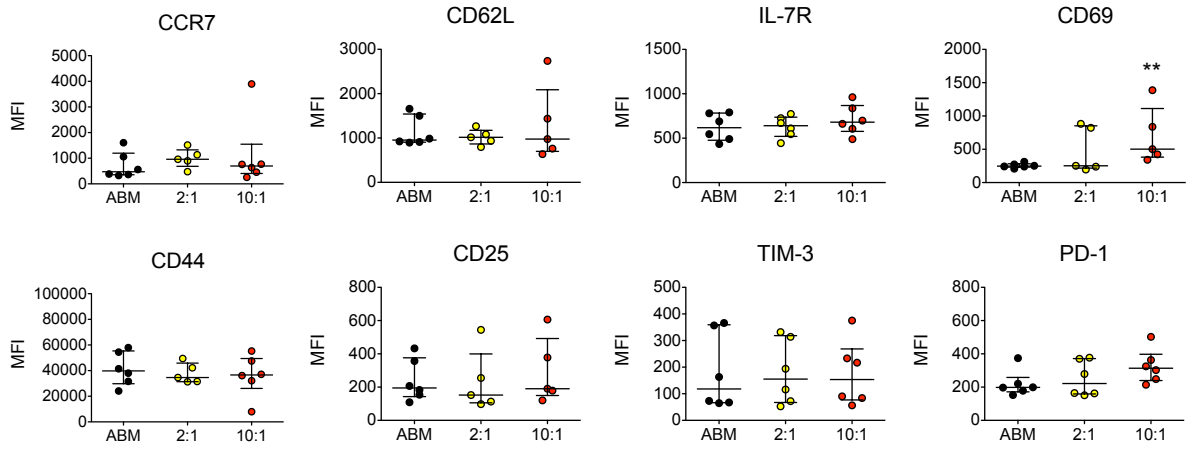
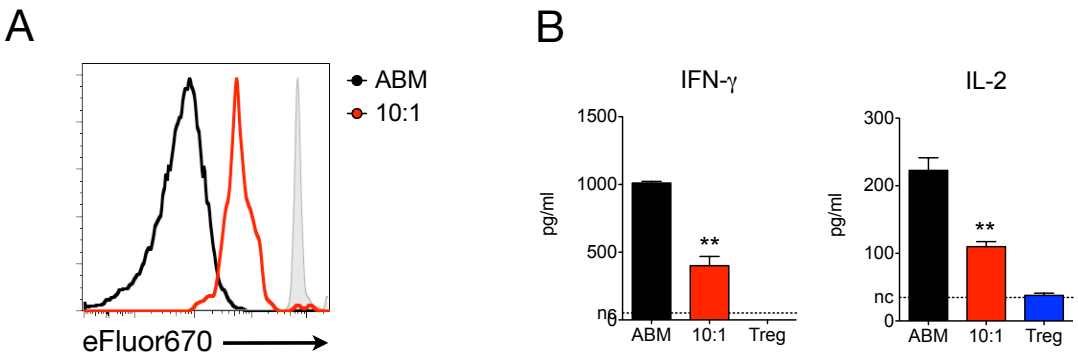


**Figure 8. Treg cells do not inhibit ABM cells activation in the LNs.** Surface expression (mean fluorescence intensity, MFI) of CCR7, CD62L, IL-7R, CD69, CD44, CD25, TIM-3 and PD-1 on ABM cells in the ABM (black), 2:1 (yellow) and 10:1 (red) groups on day 9 was determined by FACS analysis. Statistical analysis was performed using the Mann Whitney t test by comparing to the ABM group. **, $P < 0.01$. Data pooled from two independent experiments. Error bars denote SEM. n = 7

To study the effect of Treg cells on ABM cell activity we analyzed ABM T cell proliferation and cytokine production both in vitro and in vivo. In vitro, high numbers of Treg cells suppressed the proliferation of ABM cells as determined by the dilution of the eFluor670 dye (Fig. 9, A). Moreover, IFN-γ and IL-2 production by bm12-DC activated ABM cells were decreased in the presence of high numbers of Treg cells (Fig. 9, B). In agreement with these in vitro data, transferred Treg cells inhibit ABM cell proliferation in a cell number dependent manner on day 9 (Fig. 9, C) and reduced the numbers of IFN-$\gamma^+$ V$\alpha2^+$ V$\beta8^+$ ABM cells isolated from LNs of the 10:1 group (Fig. 9, D,E). Taken together, our data indicate that Treg cells inhibit the expansion of LN homing ABM cells and impair their production of effector cytokines.



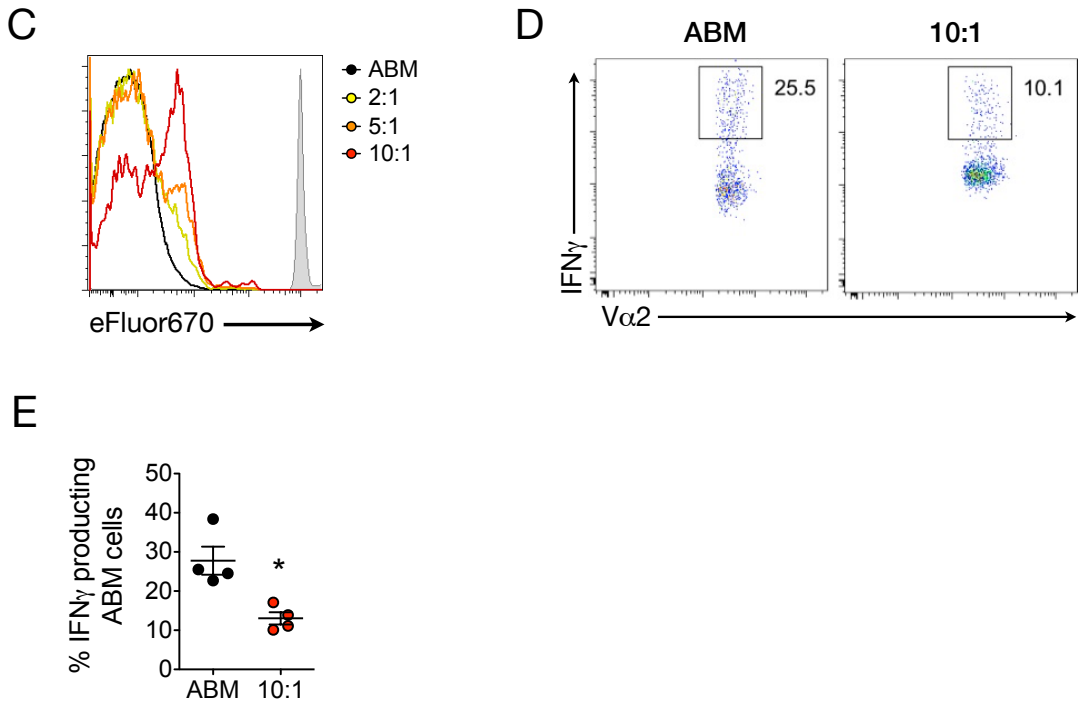



**Figure 9. Treg cells delay proliferation and impair cytokine production of ABM cells.** (A) Representative plot of the in vitro proliferation of ABM cells stimulated with anti-CD3/CD28 beads for 5 days determined by eFluor670 proliferation dye staining in the 10:1 (red) and ABM (black) group. The grey curve represents the fluorescence of undivided cells on day 0. (B) IFN-γ and IL-2 cytokines production measured by ELISA in the supernatant of ABM cells cocultured with Bm12 Rag2$^{-/-}$ LN cells in the absence (ABM, black) or presence (10:1, red) of Treg cells for 72h. Cytokine secretion by Treg alone is represented in blue. The dotted line represents the cytokine detection background. Data are representative of three independent experiments. Error bars denote SEM. (C) Representative plot of the proliferation of ABM cells determined by eFluor670 proliferation dye staining in the different groups on day 9. The grey curve represents the fluorescence of undivided cells on day 0. Data are representative of three independent experiments. (D) Representative plot of the in vivo IFN-γ production of ABM cells isolated from the LNs of the ABM (left) and 10:1 (right) group which were restimulated in vitro with PMA/ionomycin (4h) and were stained for intracellular IFN-γ. (E) Percentage of IFN-γ producing ABM cells in the different groups. n = 4. Statistical analysis was performed using the Mann Whitney t test by comparing to the ABM group. *, $P < 0.05$; **, $P < 0.01$. Error bars denote SEM.

## 4.5 Effect of adaptive immune cells on LNSCs

In recent years it has become clear that hematopoietic cells, including Treg cells, are not the sole regulators of T cell tolerance and immune responses. Indeed, non-hematopoietic LNSCs have been shown to modulate T cells responses in the LNs (105). To address a role of LNSCs in our model we first examined how T cells affect LNSCs under steady-state conditions. LNSC populations from C57BL/6, CD3e$^{-/-}$ and Rag2$^{-/-}$ mice were analyzed for markers to identify TRC (gp38$^{+}$CD31$^{-}$), LEC (gp38$^{+}$CD31$^{+}$), BEC (gp38$^{-}$CD31$^{+}$) and DNC (gp38$^{-}$CD31$^{-}$) (Fig. 10, A) subsets. The absence of T cells did not affect the numbers or frequencies of LNSC populations (Fig. 10, B).

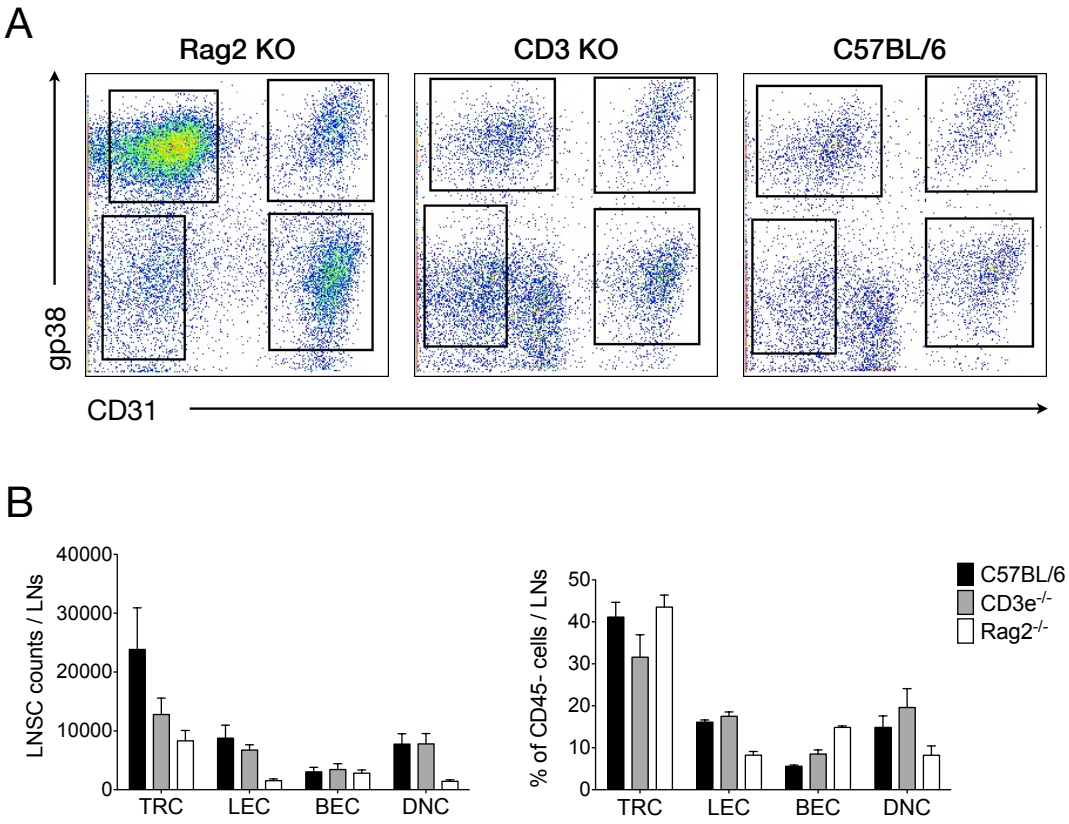
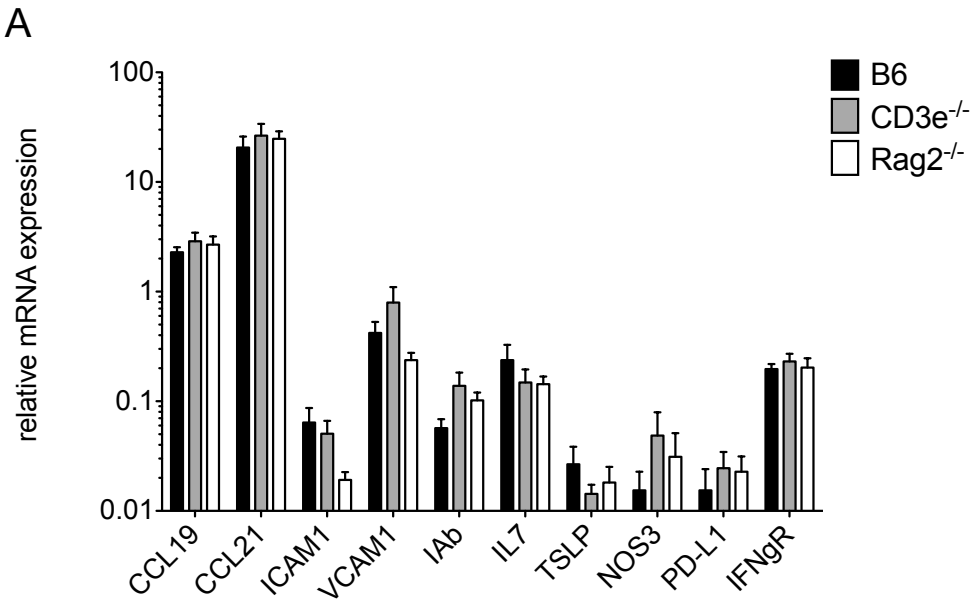



**Figure 10. C57BL/6, CD3e$^{-/-}$ and Rag2$^{-/-}$ mice have similar LNSC distribution.** (A) Representative plot of LNSC subpopulations identified in C57BL/6, CD3e$^{-/-}$ and Rag2$^{-/-}$ mice by flow cytometry analysis. CD45$^{-}$ cells were stained for gp38 and CD31 that define

TRC ($gp38^+CD31^-$), LEC ($gp38^+CD31^+$), BEC ($gp38^-CD31^+$) and DNCs ($gp38^-CD31^-$). (B) Counts (left) and frequencies (right) of TRC, LEC, BEC, and DNC in the LNs of C57BL/6 (black), $CD3e^{-/-}$ (gray), $Rag2^{-/-}$ (white). Error bars denote SEM. n = 3. Data are representative of three independent experiments.

We next addressed the transcriptional profile of the TRC subset since they are the main LNSC population to come into contact with T cells. TRCs attract naïve T cells to the LNs via the release of CCL19 and CCL21 and might interact with them via ICAM-1 and VCAM-1 (98). Furthermore, TRCs are the main producer of IL-7 and express PD-L1 and NOS upon IFN-γ stimulation. All of these molecules orchestrate effector T cell responses and were included in our RT-PCR analysis. Gene transcription of the selected genes was not affected by the absence of T or B cells under non-inflammatory conditions (Fig. 11).

In agreement with this analysis, ICAM-1 and PD-L1 surface expression was similar in all cases. In contrast, MHC class II (IAb) surface expression was dependent on the presence of T cells (Fig. 11), probably due to the absence of T cells that have been shown to require MHC class II molecules during T cell homeostasis (150, 151).



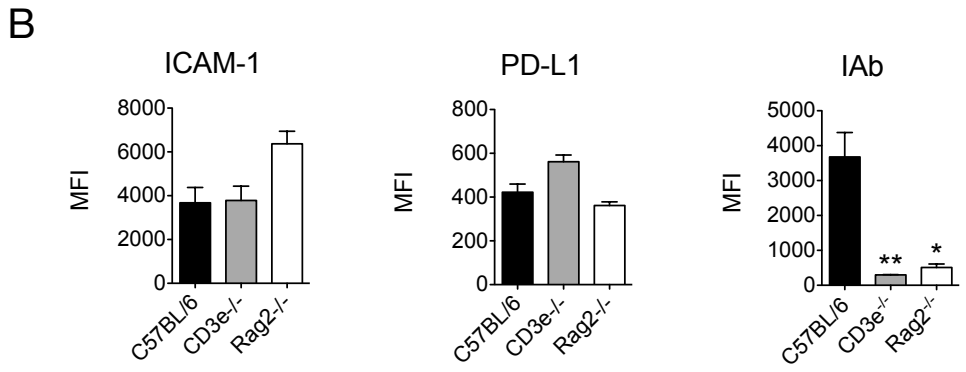



**Figure 11. IAb expression on TRCs is dependent on the presence of T cells.** (A) Quantitative RT-PCR analysis of relative mRNA expression for different genes from $CD45^{-}gp38^{+}CD31^{-}$ sorted TRCs from C57BL/6 (black), $CD3e^{-/-}$ (light gray), $Rag2^{-/-}$ (white). n = 5 (B) Cell surface expression (MFI) of ICAM-1, PD-L1 and IAb on TRC isolated from the different mice and analyzed by flow cytometry. Statistical analysis was performed using the Mann Whitney t test by comparing to the C57BL/6 group. *, $P < 0.05$; **, $P < 0.01$. Error bars denote SEM. n = 3.

## 4.6 IFN-γ modulates the biological activity of TRCs in vitro

IFN-γ and tumor necrosis factor (TNF) secreted by CD8 T cells plays a role in modulating TRC mediated suppression of T cell proliferation (104, 120). To understand the effect of IFN-γ on TRC phenotype, in vitro expanded TRCs were stimulated with IFN-γ. Culture with IFN-γ resulted in the up-regulation of the adhesion molecules ICAM-1 and VCAM-1, IAb, NOS2, NOS3 and PD-L1. In contrast, IL-7, TSLP and IFN-γ-R were not altered upon IFN-γ stimulation (Fig. 12, A). In line with the transcription analysis, IFN-γ stimulation led to the up-regulation of surface expression of IAb, PD-L1 and ICAM-1 but not VCAM-1. In order to address the dependency of these changes on TRCs IFN-γ signaling, we analyzed the up-regulation of the selected surface markers on TRCs isolated from $IFN\text{-}\gamma R^{-/-}$ mice.

Following IFN-γ stimulation IFN-γR$^{-/-}$ TRCs failed to up-regulate IAb, PD-L1 and ICAM-1 (Fig. 12, B).

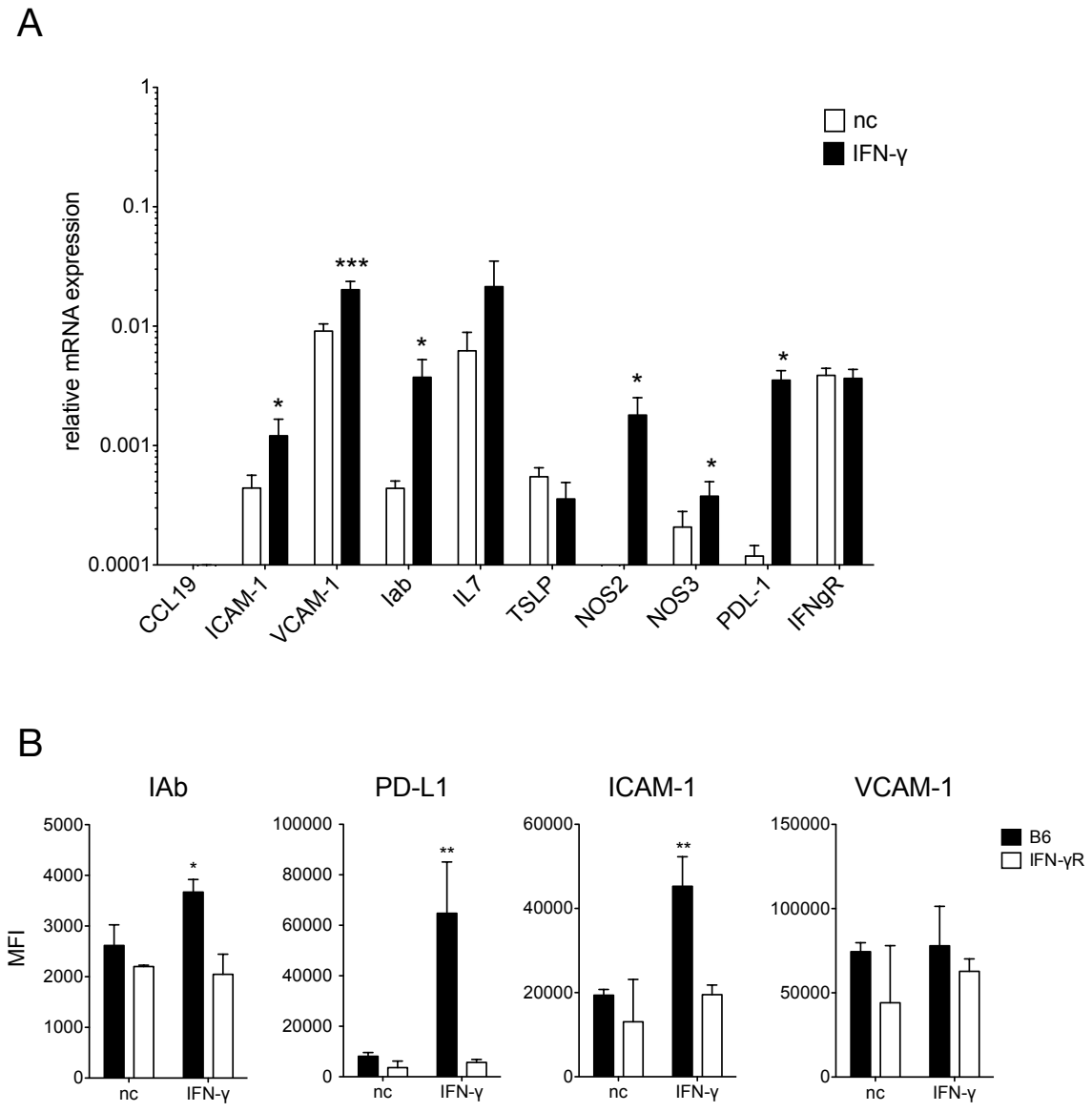
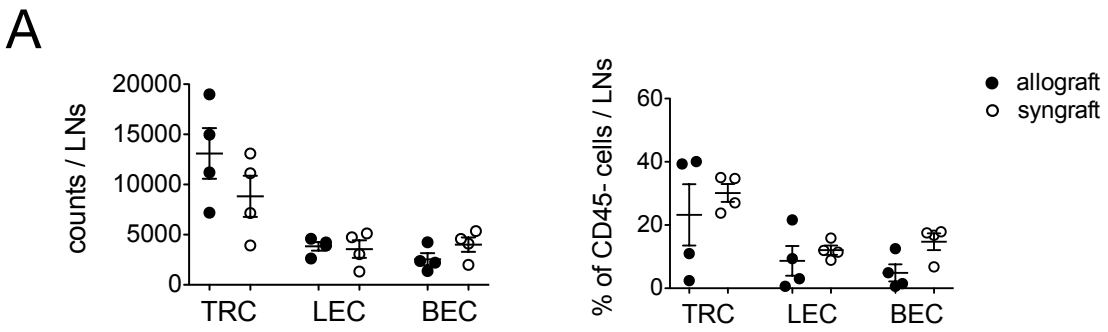



**Figure 12. TRCs up-regulate IAb, PD-L1 and ICAM-1 in an IFN-γ dependent manner.** (A) Quantitative RT-PCR analysis of relative mRNA expression of selected genes from unstimulated (white) or IFN-γ stimulated (black) TRCs cell lines 24h after in vitro culture. n ≥ 9, data pooled from 5 independent experiments. (B) Surface expression (MFI) of IAb, PD-L1, ICAM-1 and VCAM-1 on TRCs cell lines derived from C57BL/6 mice (black) or IFN-γR$^{-/-}$ mice

stimulated with or without IFN-γ. n = 4. Statistical analysis was performed using an unpaired two-tailed student's t test. Error bars denote SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

## 4.7 Modulation of TRCs during allo-responses

To study the modulation of TRCs gene expression and phenotype during acute skin-transplantation, we isolated TRCs from syngraft-transplanted C57BL/6 mice and compared them to allograft-transplanted mice. TRC as well as LEC and BEC numbers and frequencies were similar in both syngrafted and allografted mice (Fig. 13, A). TRCs isolated from allograft transplanted mice exhibited higher gene transcription of ICAM-1, VCAM-1, IAb, IL-7, TSLP and PD-L1 compared with TRCs isolated from syngeneic grafted mice (Fig. 13, B). In contrast to these mRNA data, no differences of surface marker expression of ICAM-1, VCAM-1, IAb and PD-L1 were observed (Fig. 13, C). We cannot exclude that the discrepancy between mRNA and surface protein levels is not a consequence of the digestion steps needed to isolate TRCs from LNs which may affect the expression of these molecules.



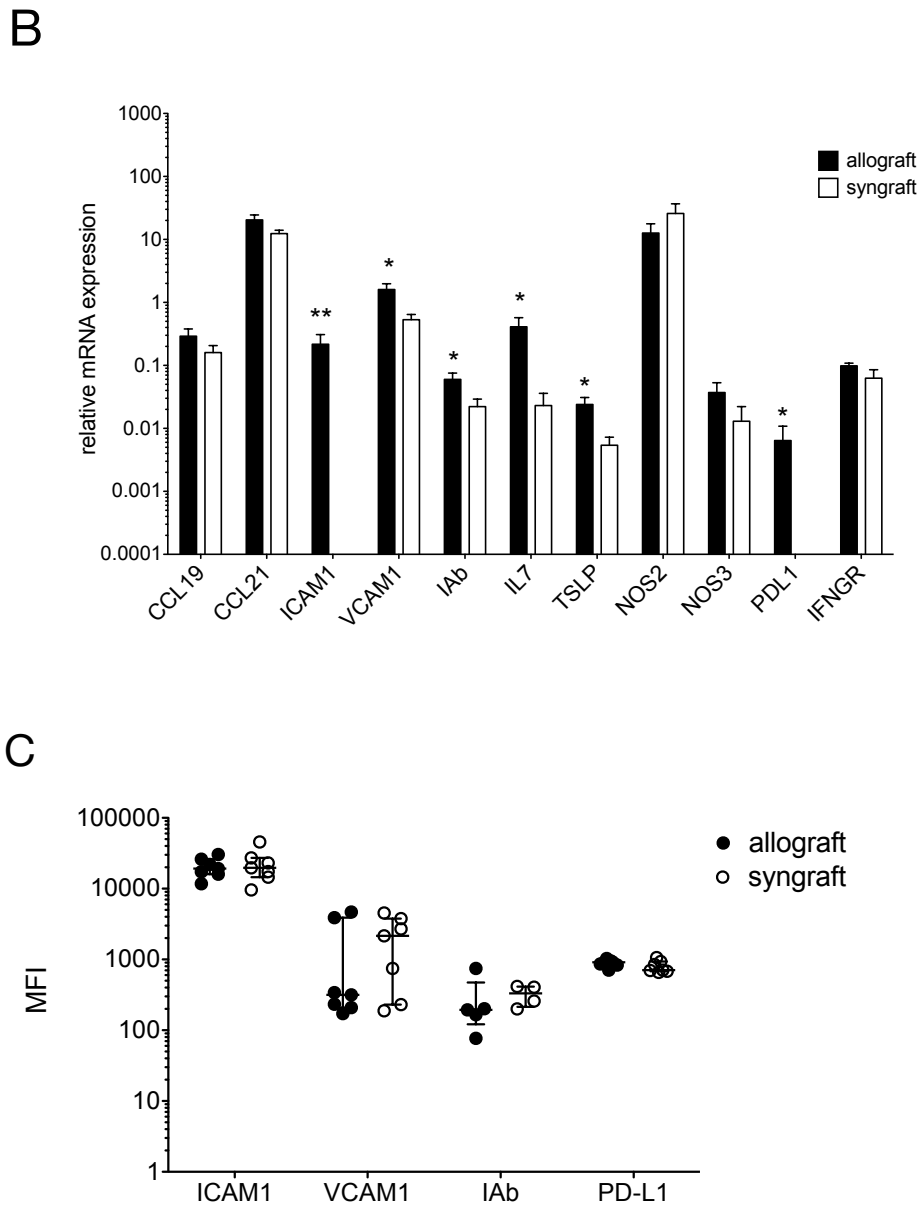
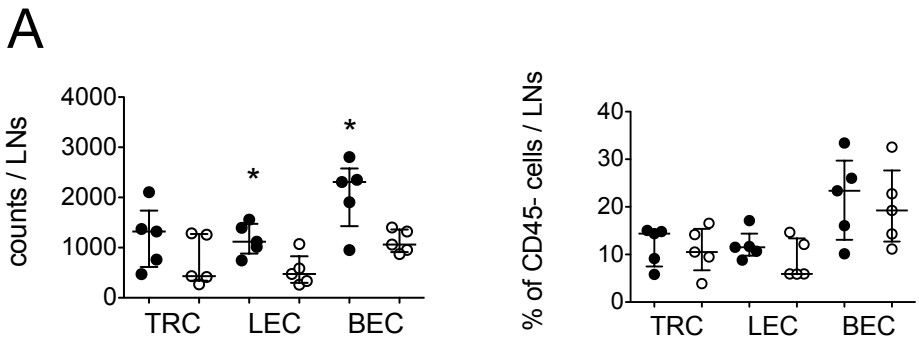



**Figure 13. Allograft response modifies TRC transcription profile.** (A) Counts and frequencies of TRCs, LECs and BECs of allograft (black) or syngraft (white) transplanted C57BL/6 mice and analyzed by FACS after CD45, CD31 and gp38 staining 7 days after transplantation. n = 4. (B) Quantitative RT-PCR analysis of relative mRNA expression for different genes from $CD45^{-}gp38^{+}CD31^{-}$ sorted TRCs from Bm12-allograft (n = 6, black) or syngraft (n = 7, white) transplanted C57BL/6 mice. The expression of selected genes was normalized on β-actin expression. Error bars denote SEM. (C) Surface expression of selected molecules was determined by FACS analysis for the different LNSC subsets 7 days

after transplantation. Error bars denote interquartile range. n = 4. Statistical analysis was performed using the Mann Whitney t test. *, $P < 0.05$; **, $P < 0.01$.

To investigate the role of IFN-γ in the modulation of LNSC activity during alloresponses, we transplanted IFN-$\gamma^{-/-}$ mice with syngraft or Bm12-allograft and analyzed LNSCs 7 days after transplantation. The number of TRCs was similar, while LEC and BEC numbers were higher during alloresponses. However the LNSC frequencies were similar for all the populations. The differences in mRNA expression between TRCs isolated from allogeneic and syngeneic transplanted mice were abolished in IFN-$\gamma^{-/-}$ recipients (Fig. 14).

Our data underscore the importance of IFN-γ signaling on the transcription profile of adhesion and survival molecules in TRCs during acute allogeneic immune responses.



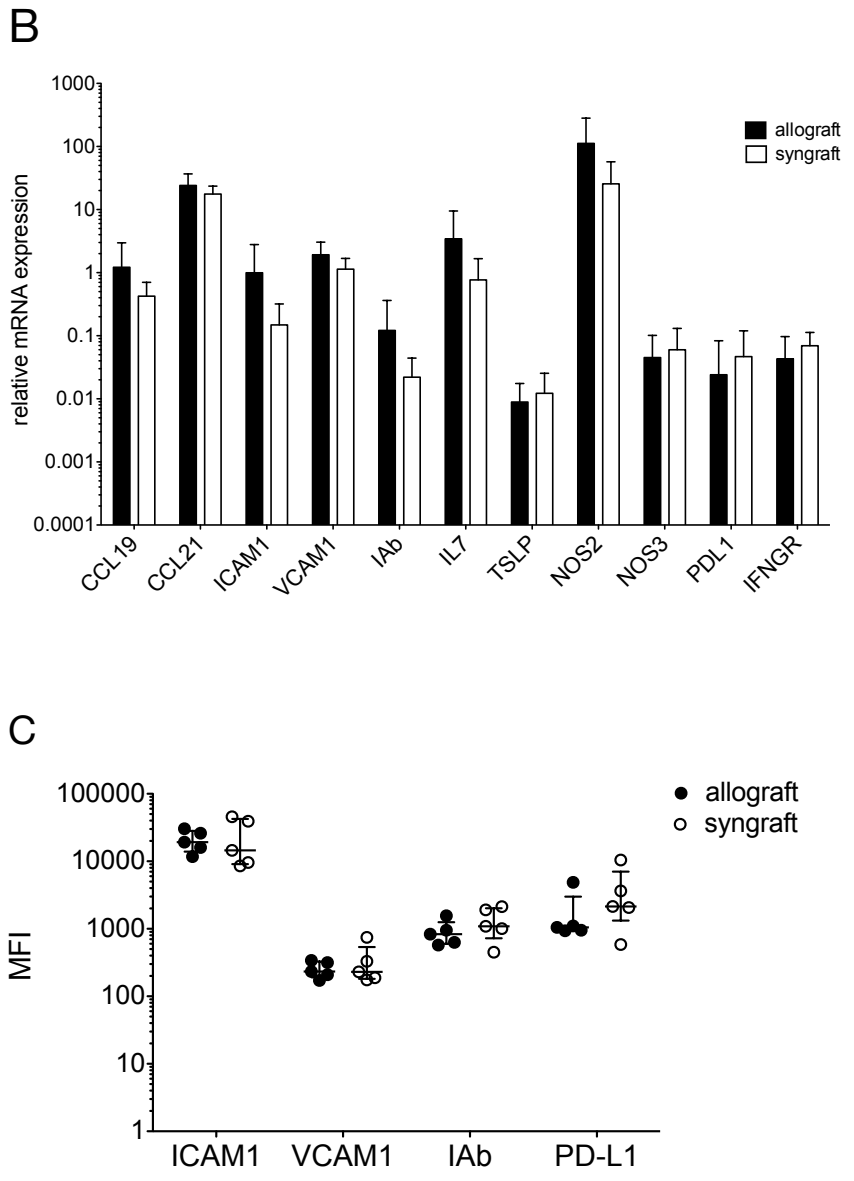



**Figure 14. Allograft response does not modify TRC transcription profile in the absence of IFN-γ.** (A) Counts and frequencies of TRCs, LECs and BECs of allograft (black) or syngraft (white) transplanted IFN-$\gamma^{-/-}$ mice and analyzed by FACS after CD45, CD31 and gp38 staining 7 days after transplantation. (n = 5) (B) Quantitative RT-PCR analysis of relative mRNA expression for different genes from $CD45^{-}gp38^{+}CD31^{-}$ sorted TRCs from Bm12-allograft (n = 6, black) or syngraft (n = 6, white) transplanted IFN-$\gamma^{-/-}$ mice. The expression of selected genes was normalized on β-actin expression. (C) Surface expression of selected molecules was determined by FACS analysis for the different LNSC subsets 7

days after transplantation. n = 5. Statistical analysis was performed using the Mann Whitney t test. *, P < 0.05.

## 4.8 Modulation of TRCs during Treg-mediated allo-tolerance

To investigate the response of TRCs during Treg-mediated allo-tolerance, we analyzed TRC numbers, transcription profile, and phenotype in Rag2$^{-/-}$ Bm12-transplanted mice. TRCs were compared after adoptive transfer of naïve ABM with or without Treg cells on day 6 (Fig. 15, A) and day 9 (Fig. 15, B). The transfer of ABM or Treg cells alone or in different Treg:ABM ratios (2:1 and 10:1) did not affect the total number or frequency of TRCs, or BEC and LEC at either time points (Fig. 15). In addition, the proliferation of LNSC subsets was not affected by the presence of Treg cells (2:1 group) in vivo on day 6 (Fig. 15, C).

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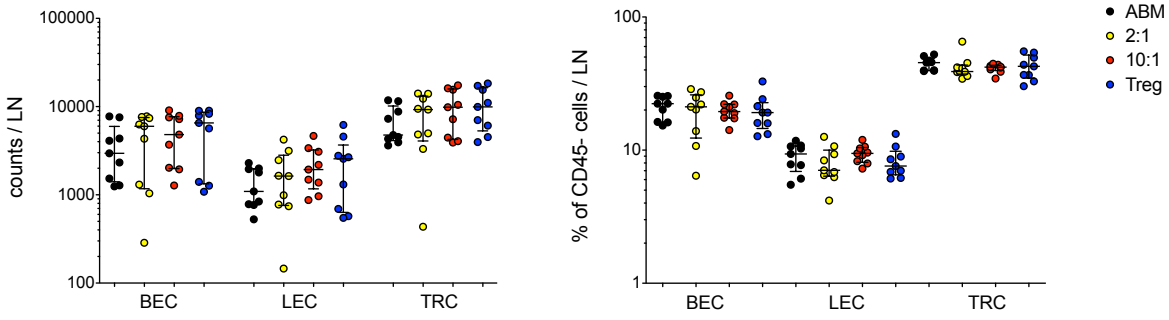


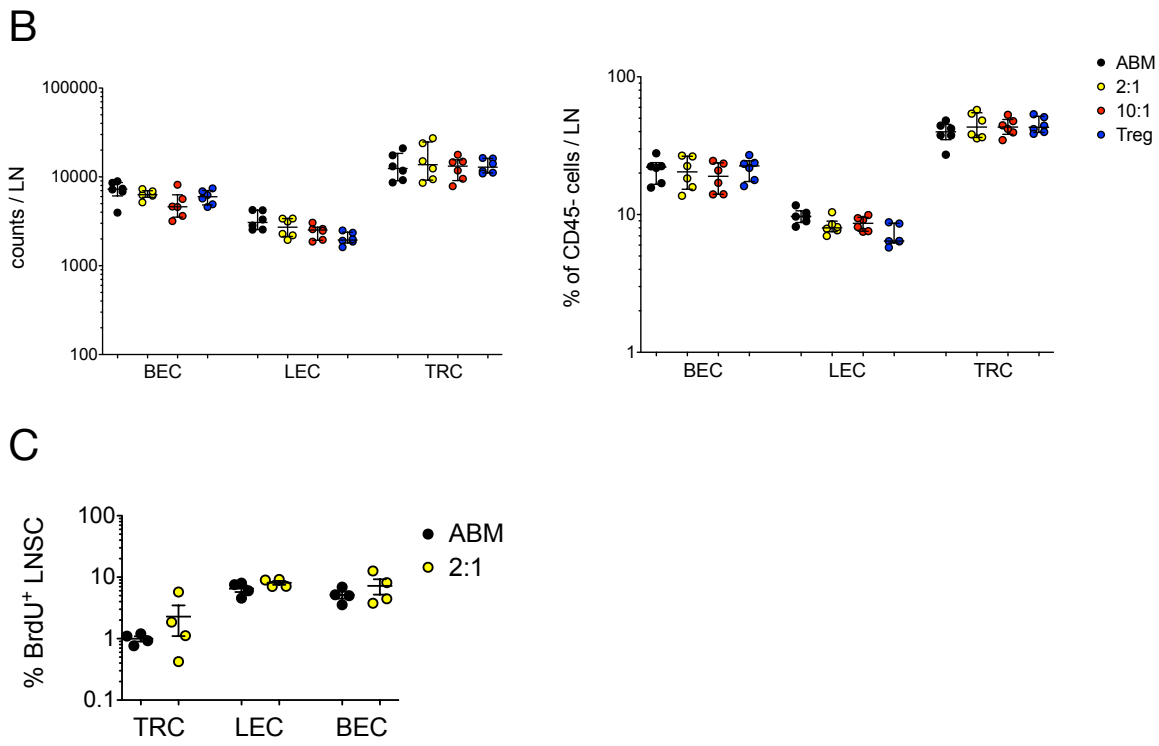



**Figure 15. Co-transfer of Treg cells does not influence number and frequencies TRCs.** Counts (left) and frequencies (right) of TRCs in the LNs of Bm12-transplanted Rag2$^{-/-}$ mice on day 6 (n = 9) (A) and day 9 (n = 6) (B) in ABM (black), 2:1 (yellow), 10:1 (red) and Treg (blue) group analyzed by flow cytometry. (C) LNSCs in vivo proliferation was determined by bromdesoxyuridin (BrdU) incorporation in the different groups 6 days after adoptive T cells transfer. Differences were not significant using the Mann Whitney t test.

Six days after adoptive T cells transfer, the transcriptional analysis on TRCs showed only a few differences in the gene expression profile among the groups. In the 10:1 group IAb and PD-L1 were significantly higher compared to the ABM group and baseline expression (dotted line) (Fig. 16, A). mRNA levels of chemokines, adhesion molecules, cytokines, cytokines receptors and growth factors were not affected by different ratios of Treg cells (Fig. 16, A). Surface expression of IAb, PD-L1, ICAM-1 was similar in all groups, whereas VCAM-1 was significantly lower expressed on TRCs isolated from the Treg group compared to the one isolated from the ABM group (Fig. 16, B).

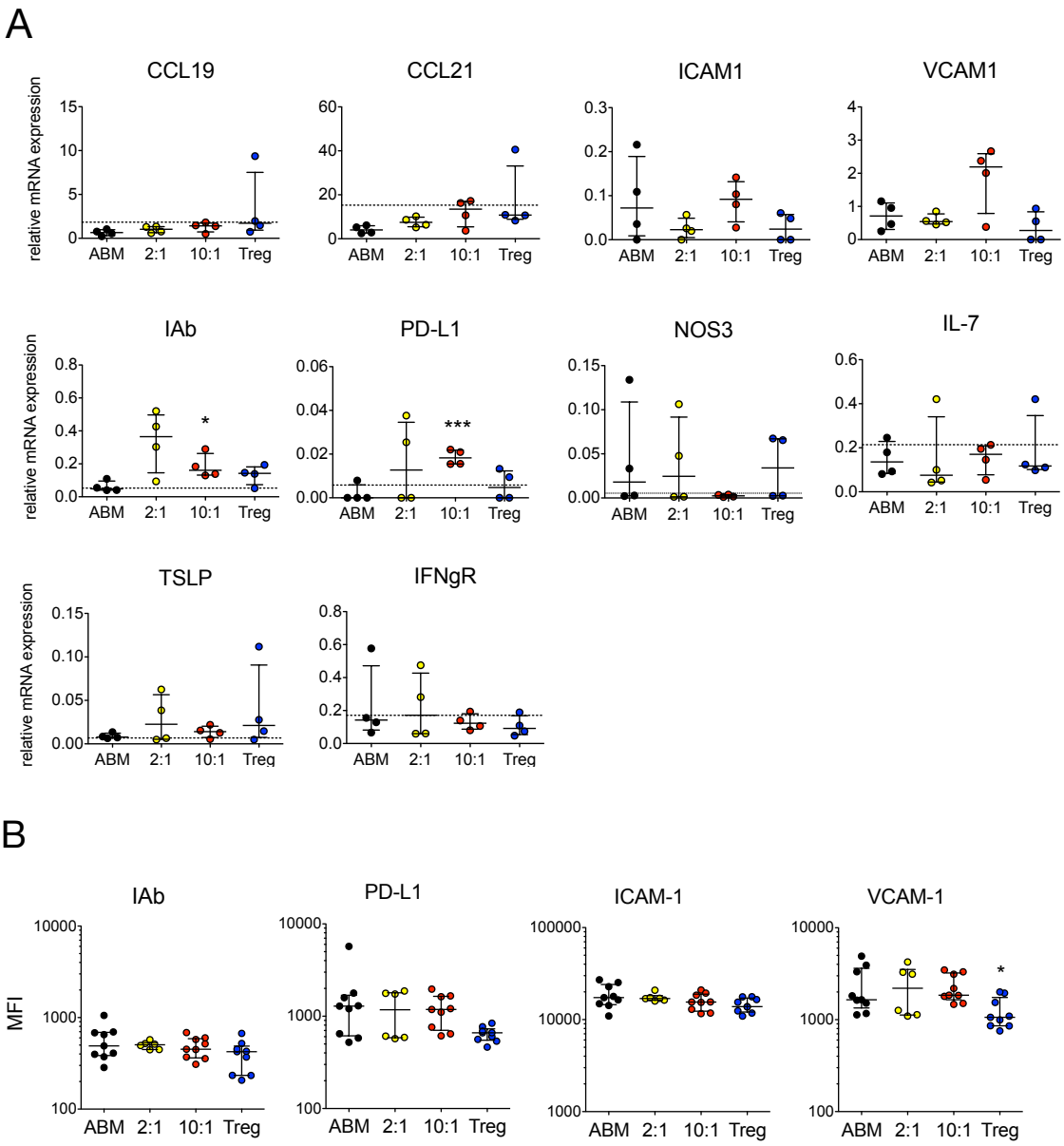
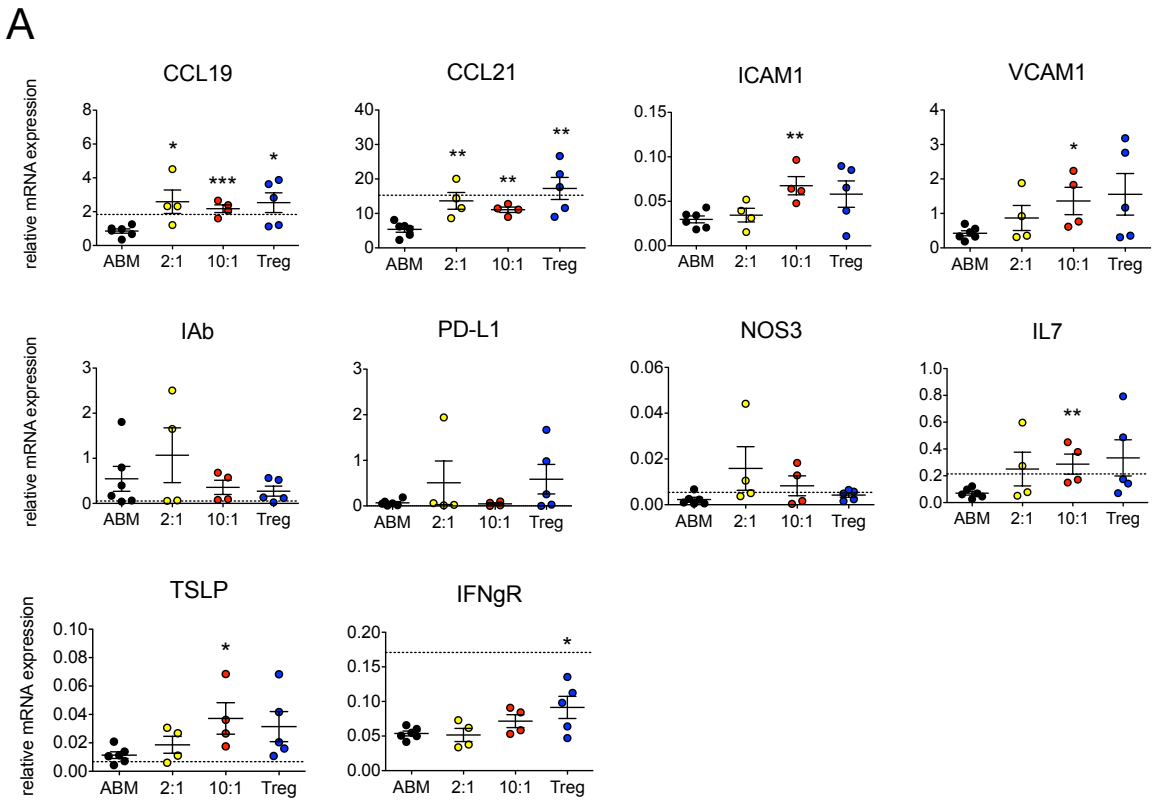



**Figure 16. Co-transfer of Treg cells does not influence TRCs activity on day 6.** (A) Quantitative RT-PCR analysis of relative mRNA expression for different genes from $CD45^{-}gp38^{+}CD31^{-}$ sorted TRCs from Bm12-allograft transplanted $Rag2^{-/-}$ mice (n = 4) on day 6. The expression of selected genes was normalized on β-actin expression. Dotted line represents mRNA levels of TRCs isolated from transplanted not adoptively transferred $Rag2^{-/-}$ mice. (B) Mean fluorescence intensity of selected markers in the different groups was measured by FACS analysis. (n ≥ 6). Statistical analysis was performed using an unpaired two-tailed student's t test. Error bars denote SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Nine days after T cell transfer, the expression of ICAM-1, VCAM-1, IAb, PD-L1 and TSLP was induced in all groups, while the expression of CCL19 and CCL21, IL-7 and IFN-γR was maintained or lower compared to non-injected controls (baseline) (Fig. 17, A). mRNA levels of CCL19 and CCL21 were higher after Treg cell transfer in different ratios (2:1, 10:1, Treg alone) than in the ABM group, suggesting that Treg cells maintain the chemokine and cytokine expression of TRCs (Fig. 17, A). The mRNA levels of ICAM-1, VCAM-1, IL-7 and TSLP were higher in the 10:1 group and IAb, PD-L1, NOS3 and IFN-γR transcripts were comparable in all groups (Fig. 17, A). In contrast to these transcriptional data, surface expression of ICAM-1 was similar and VCAM-1 was down regulated in the presence of Treg cells (Fig. 16, B). Consistent with the mRNA levels, PD-L1 and IAb surface expression was comparable in all groups (Fig. 16, B).



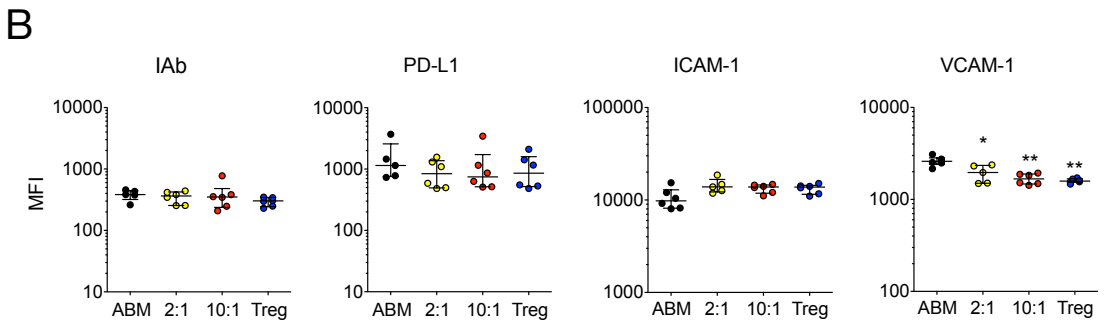



**Figure 17. Co-transfer of Treg cells modifies transcription profile of TRCs on day 9.** (A) Quantitative RT-PCR analysis of relative mRNA expression for different genes from $CD45^{-}gp38^{+}CD31^{-}$ sorted TRCs from Bm12-allograft transplanted $Rag2^{-/-}$ mice (n = 4) on day 9. The expression of selected genes was normalized on β-actin expression. Dotted line represents mRNA levels of TRCs isolated from transplanted not adoptively transferred $Rag2^{-/-}$ mice. (B) Mean fluorescence intensity of selected markers in the different groups was measured by FACS analysis. ($n \geq 6$). Statistical analysis was performed using an unpaired two-tailed student's t test. Error bars denote SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Taken together, transcription of the chemoattractans CCL19 and CCL21 is up-regulated by Treg cells regardless of the establishment of tolerance, while the transcription of adhesion molecules ICAM-1, VCAM-1, IL-7 and TSLP were only up-regulated during establishment of Treg mediated tolerance (10:1 group).

## 4.9 Activation of Treg cells during tolerance influences their function

To assess whether changes in the transcription profile of TRCs correlates with the activation status of Treg cells in the different groups, we analyzed Treg cell phenotype on day 6 and day 9 (Fig. 18).

On day 6, Treg cells isolated from the 10:1 group were expressing similar surface level of CD25, CD44, CD62L, CD69, CCR7, Tim 3, and PD-1 compared to the Treg isolated from the Treg only group. We found higher IL-7R expression on Treg cells isolated from the 2:1 group on day 6 (Fig. 18, A). On day 9, Treg cells from the different groups were similar in CD44, CD62L, CD69, IL-7R, and CCR7 expression, whereas suppression of alloresponses (10:1 group) increased CD25 and TIM 3 compared to Treg cells from Treg only injected mice. Expression of PD-1 was lower in the 2:1 than in the 10:1 and Treg group (Fig. 18, B).

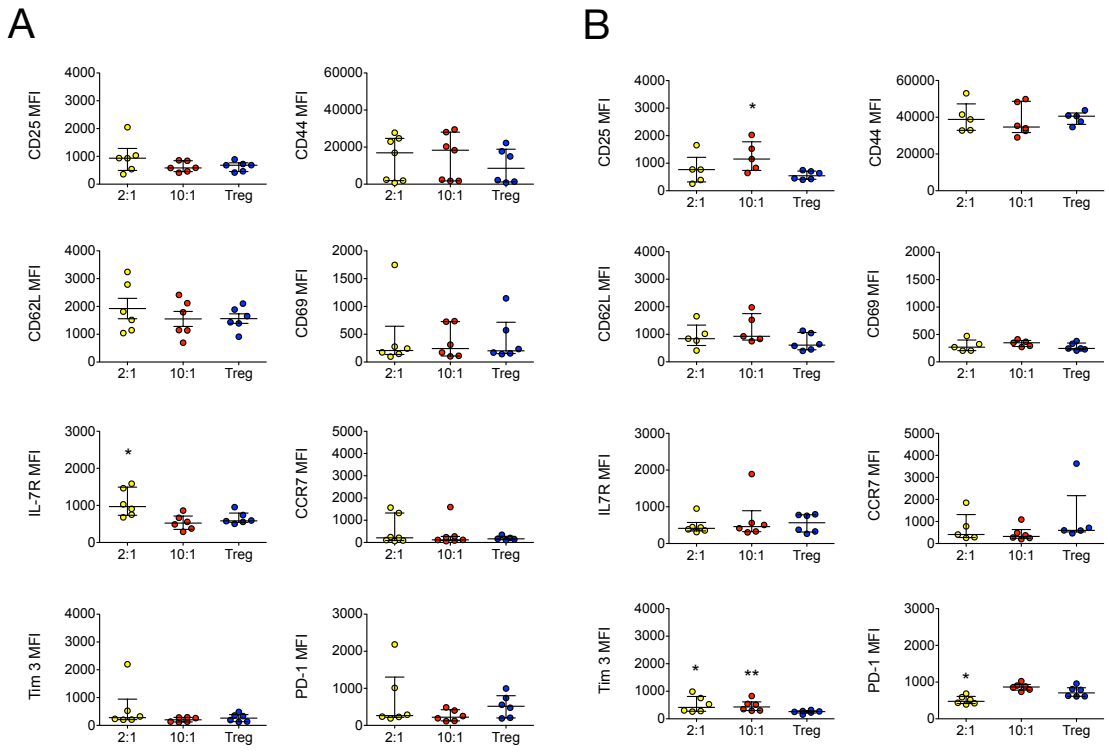



**Figure 18. Treg cells have higher CD25, TIM-3 and PD-1 during suppression on alloresponses.** Mean fluorescence intensity of different surface molecules on FoxP3eGFP$^{+}$ Treg cells isolated from the 2:1 (yellow), 10:1 (red) and Treg (blue) group at (A) day 6 (n = 6) and (B) day 9 (n ≥ 5). For both time point, pooled data of two independent experiments. Statistical analysis was performed using the Mann Whitney t test. *, $P < 0.05$; **, $P < 0.01$. Error bars denote interquartile range.

## 4.10 Effect of IL-7 on Treg cells

Given our observation that IL-7 transcription in TRCs was higher during Treg-mediated skin graft tolerance compared to the ABM group (Fig. 17, A), we next analyzed the molecular basis of IL-7R signaling on Treg cells in vitro. In agreement with previous reports (86-88), we confirmed constitutive expression of IL-7R on Treg cells and IL-7R down regulation following treatment with IL-7 (Fig. 19, A). In addition, IL-7 induced STAT5 phosphorylation (Fig. 19, B) and increased survival of naïve Treg cells in vitro (Fig. 19, C). Moreover, Treg cells had higher surface CD25 expression in the presence of IL-7, while CD62L, CD69, PD-1 and TIM-3 surface expression were similar (Fig. 19, D).

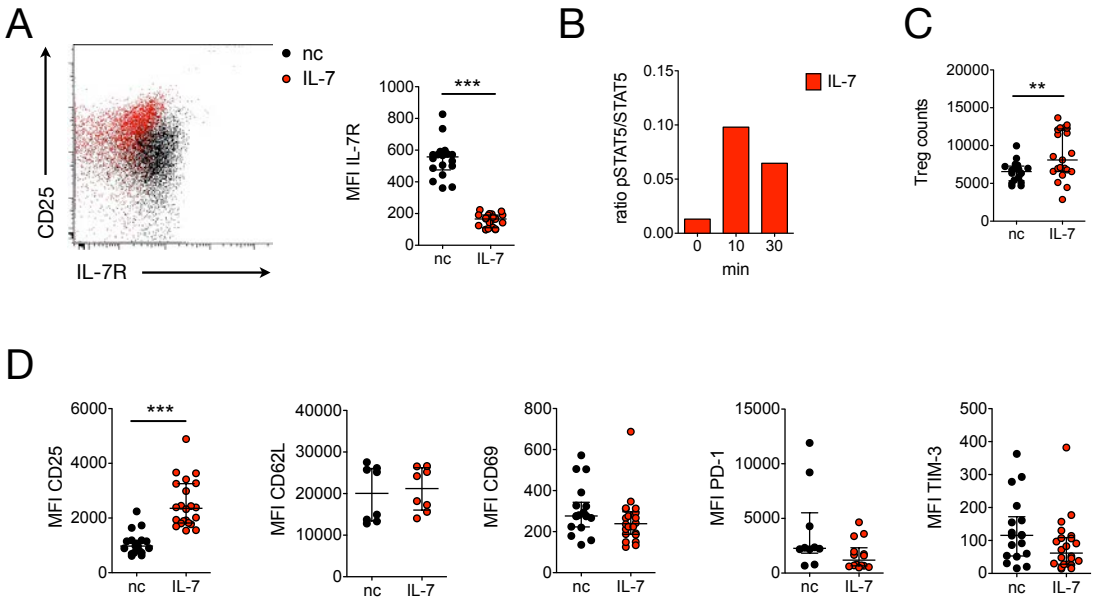



**Figure 19. IL-7R signaling on Treg cells enhances survival and CD25 expression.** (A) Representative plot of IL-7R and CD25 expression (left) and mean fluorescence intensity of IL-7R (right) on unstimulated (black) or IL-7-stimulated (red) naïve Treg cells analyzed by flow cytometry after 24h of in vitro culture. n ≥ 18 pooled data from 4 different experiments. (B) Ratio of phosphorylated STAT5 on STAT5 after 0, 10 and 30 minutes IL-7 stimulation. (C) Cells counts and phenotype (D) of unstimulated (black) or IL-7-stimulated (red) naïve Treg cells analyzed by flow cytometry after 24h of in vitro culture. n ≥ 18 pooled data from 4

different experiments. Statistical analysis was performed using an unpaired two-tailed student's t test. Error bars denote interquartile range. **, P < 0.01; ***, P < 0.001.

To investigate the effect of IL-7 during activation, we stimulated Treg cells with CD3/CD28 Ab-coated beads for 4 days. Activated Treg cells were present in higher numbers after IL-7 stimulation (Fig. 20, A) and responded in a similar fashion by downregulating IL-7R and increasing CD25 expression (Fig. 20, B). By comparing IL-7 with IL-2 stimulated Treg cells, we found that IL-2 strongly increased Treg numbers (Fig. 20, A) and CD25 expression, which was not further enhanced by the addition of IL-7 (Fig. 20, B). Moreover, TIM-3 expression was upregulated on activated Treg cells by IL-2, while PD-1 expression was unaltered by the cytokine stimulations.

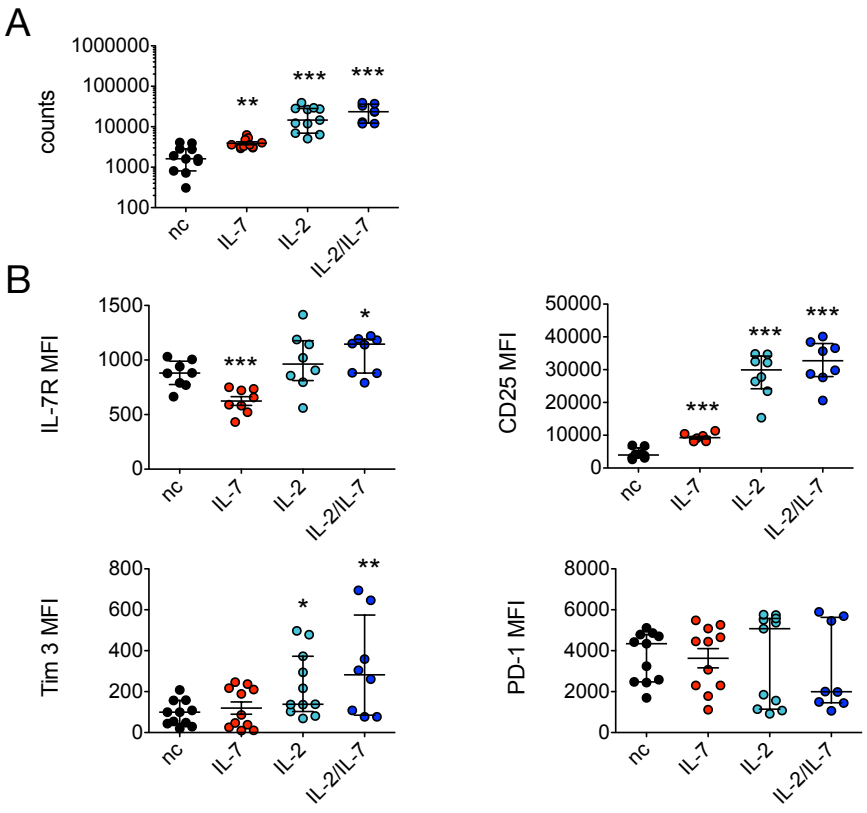



**Figure 20. Activated Treg cells can sense IL-7.** (A) Cell counts and phenotype (B) of unstimulated (black) or IL-7- (red), IL-2- (light blue) and IL-2/IL-7 (blue)-stimulated anti-

CD3/CD28 activated Treg cells analyzed by flow cytometry after 4 days of in vitro culture. n ≥ 11 pooled data from 4 different experiments. Statistical analysis was performed using an unpaired two-tailed student's t test. Error bars denote interquartile range. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

The CD25 molecule is constitutively expressed on the surface of Treg cells and contributes to Treg mediated suppression by sequestering IL-2 produced by ABM effector cells. To examine the effect of IL-7 on Treg activity, we studied the ability of Treg cells to suppress ABM cells proliferation following IL-7 stimulation in vitro. In the absence of Treg cells, the MFI of the proliferation-dye on ABM cells was higher by the addition of exogenous IL-7, suggesting that ABM cells alone are less proliferating in the presence of IL-7. Treg cells, in a 2:1 situation, were increasing the MFI of ABM cells which was higher by the addition of exogenous IL-7 in vitro (Figure 21), suggesting that IL-7-mediated CD25 upregulation on Treg cells enhance their ability to suppress proliferation.

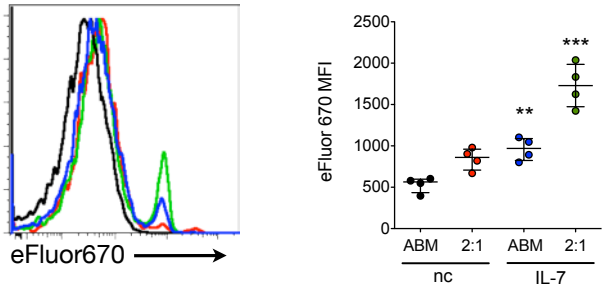
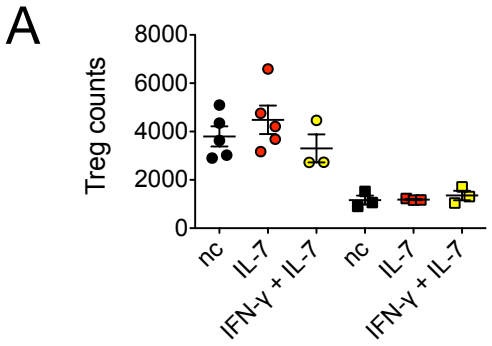



**Figure 21. IL-7 stimulation enhances Treg-mediated suppression of ABM cells.** (A) Proliferation of eFluor670 stained ABM cells measured after 5 days of anti-CD3/CD28 beads activation in vitro without hrIL-2. ABM cells were cultured alone (black) or together with Treg cells in a 2:1 situation (red) without any additional stimulation or with 50ng/ml IL-7 (ABM, blue and 2:1, green). (B) Proliferation presented as MFI of proliferation dye eFluor670. Data are represented as median with interquartile range. n = 4. Statistical analysis was performed using an unpaired two-tailed student's t test. **, $P < 0.01$; ***, $P < 0.001$.

## 4.11 Interaction of Treg cells and TRCs in vitro

Finally, we investigated whether exposure to IL-7 in addition to the contact with TRCs modulate naïve (Fig. 22, circles) and activated (Fig. 22, squares) Treg cells. Since TRC cell lines have been shown to express only low levels of IL-7 (104), we added exogenous IL-7 to the culture. Furthermore, to mimic the 10:1 situation in vivo, we added IFN-γ and IL-7 to the Treg in vitro. The IL-7-mediated increase in naïve and activated Treg cell counts (Fig. 22) was abolished in the presence of TRCs and was not further changed in the presence of IFN-γ (Fig. 22, A). IL-7R downregulation and the CD25 up-regulation on naïve but not on activated Treg cells were maintained in the presence of TRCs (Fig. 22, B). CD62L was downregulated on naïve Treg cells after IFN-γ + IL-7 stimulation, while CD69 and TIM-3 were not differently expressed by the different cytokine stimuli. Beads activated Treg cells showed CD62L downregulation, and upregulation of CD69, CD25 and TIM-3, independent of cytokine stimulation.



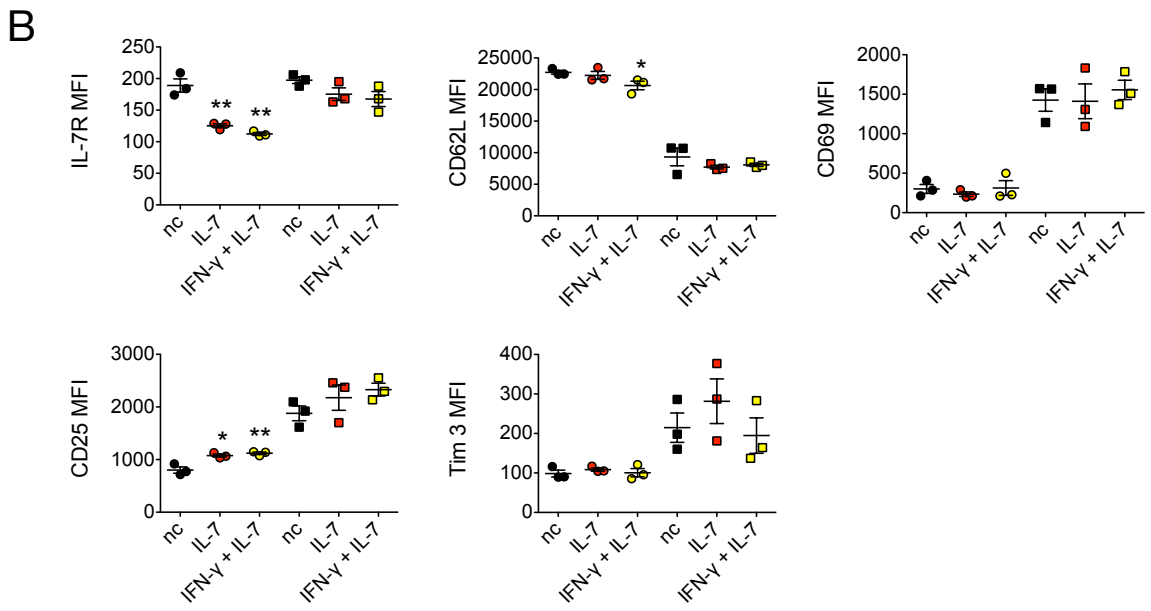



**Figure 22. Effect of IL-7 on Treg cells in the presence of TRCs.** (A) Cell counts and phenotype (B) of naïve (circles) or anti-CD3/CD28 activated (quares) Treg cells unstimulated (black) or IL-7- (red), IFN-γ/IL-7 (yellow)-stimulated analyzed by flow cytometry after 4 days of in vitro coculture with TRC cell line. n ≥ 3, data representative of two independent experiements. Statistical analysis was performed using an unpaired two-tailed student's t test. Error bars denote SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

The suppression of ABM proliferation by Treg cells in the presence of TRCs was enhanced by the addition of exogenous IL-7 (Fig. 23).

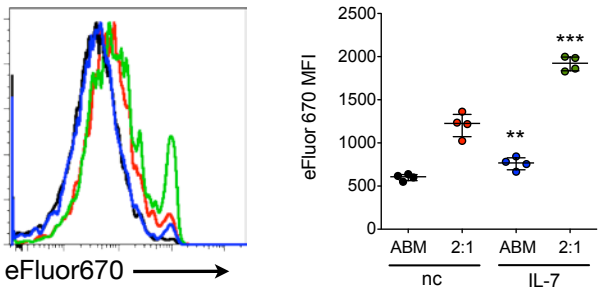
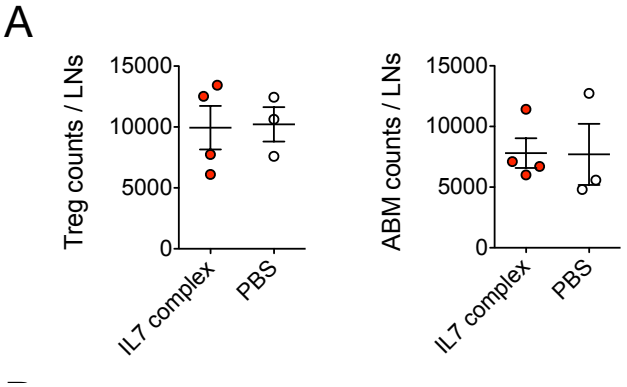



**Figure 23. IL-7 stimulation enhances Treg-mediated suppression of ABM cells on TRCs.** (A) Proliferation of eFluor670 stained ABM cells measured after 5 days of anti-

CD3/CD28 beads activation in vitro without hrIL-2. ABM cells were cultured alone (black) or together with Treg cells in a 2:1 situation (red) without any additional stimulation or with 50ng/ml IL-7 (ABM, blue and 2:1, green). (B) Proliferation presented as MFI of proliferation dye eFluor670. Data are represented ad median with interquartile range. n = 4. Statistical analysis was performed using an unpaired two-tailed student's t test. **, P < 0.01; ***, P < 0.001.

## 4.12 Contribution of IL-7 to Treg-mediated tolerance

To show the effect of IL-7 in the establishment of Treg-mediated tolerance, we injected 2:1 bm12-transplantated mice with IL-7 complex generated with IL-7 and the anti-IL-7 Ab M25. We used IL-7 complex instead of IL-7 alone because it was shown to increase the life span and enhance the biological activity of IL-7 in vivo (152), as it was reported for the IL-2 complex (153). Treated mice did not increase in counts (Fig. 24, A) and frequency of Treg and ABM cells (data not shown) and rejected the skin graft within 16 days similar to PBS control (Fig. 24, B). In addition, IL-7R down-regulation and CD25 up-regulation were not detected in either Treg cells or ABM cells (data not shown).



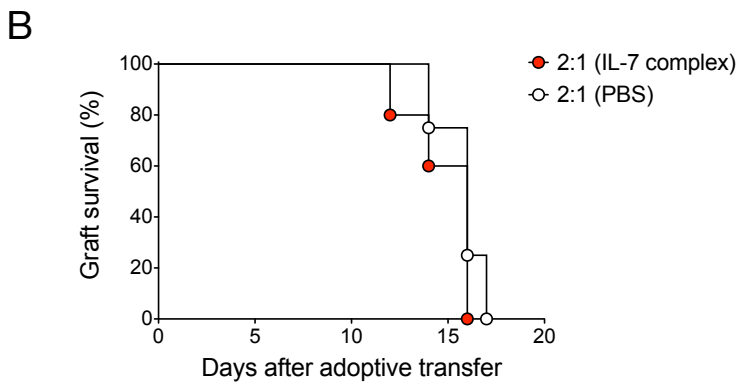



**Figure 24. IL-7 complex injection does not promote tolerance in 2:1 mice.** (A) Counts of Treg cells (left) and ABM cells (right) found in the skin dLN of allograft transplanted Rag2$^{-/-}$ mice adoptively transferred with 2:1 Treg:ABM cells ratio and treated with IL-7-complex (formed with 1µg mrIL-7 and 5µg M25 Ab per mouse) or with PBS. Mice were treated starting from day 0 for 5 times at 3 days interval. (B) Graft survival is displayed as Kaplan-Meier plot. The difference between the groups is not statistically significant using Log-rank (Mantel Cox) test.

Consequently, we blocked IL-7 expression by injecting the anti-IL-7 Ab M25 (154) (47). IL-7 blocking treatment started before (day -3) or after (day 6) adoptive T cells transfer induced graft rejection in 1/6 and 2/6 10:1 mice respectively until day 21 (Fig. 25). At day 21 mice were sacrificed to analyze the T cells phenotype. Both Treg cells and ABM cells were only slightly decreased in numbers and the expression of IL-7R and CD25 was similar to PBS control. These data suggest that addition or blocking of IL-7 in vivo does not affect Treg counts, CD25 expression and Treg-mediated tolerance. This is inconsistent with the in vitro data, however may reflect specific in vivo issues such as successful delivery to the LNs and persistence there.

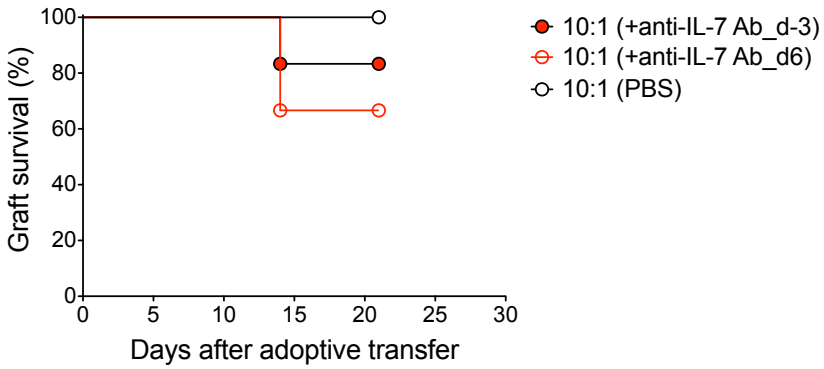



**Figure 25. Blocking IL-7 does not inhibit tolerance in 10:1 mice.** Allograft transplanted Rag2$^{-/-}$ mice adoptively transferred with 10:1 Treg:ABM cells ratio and treated with anti-IL-7 Ab M25 (0.5µg/kg) or PBS (n = 6). One treatment started 3 days before (n = 6) or 6 days (n = 6) after adoptive T cells transfer for 5 times at 3 days interval. Graft survival is displayed as Kaplan-Meier plot. The difference between the groups is not statistically significant using Log-rank (Mantel Cox) test.

# 5 Discussion

Successful organ transplantation depends on the establishment of tolerance to non-self antigens. To reach this goal patients must undergo long-term treatment with immunosuppressive drug combinations, which penalize the entire immune system and inflict many unwanted side effects including an increased risk of infections and tumors. It is therefore of primary importance to design new treatments which favor the development of allo-antigen specific tolerance. Nowadays, two main tolerizing protocols are being tested in clinical trials: 1) blocking the activation of Ag-specific T cells by injection of antibodies against co-stimulatory molecules and 2) transfusion of ex vivo expanded Treg cells. Mice tolerant to skin grafts through co-receptor blockade contain Treg cells within the allograft, which prevent graft rejection. Treg cells have been shown to convert naïve T cells into tolerant cells in a process known as *infectious tolerance* (155). Although many mouse models have been generated to study allograft tolerance, recipient mice must be continuously treated with specific depleting agents or co-stimulation blocking antibodies to achieve long-term tolerance. These treatments have many non-specific effects, which make results difficult to interpret. Moreover, specific cell-cell interactions are difficult to study due to various parameters changing during the transplant rejection process.

Our animal model has the advantage to dissect in vivo the activity of different cellular component, like Treg, dendritic cells, effector T cells and stroma cells at different time points. CD4 T cells (ABM cells), recognizing the bm12 mutation in the antigen presenting groove of the MHC class II molecule, generate an alloresponse against a major MHC mismatched antigen and cause graft rejection. The cotransfer of polyclonal Treg cells in combination with naïve bm12-specific CD4 effector T cells (ABM cells) allowed us to

investigate the immunomodulatory function of Treg cells and their interaction with other cells at the site of graft rejection and T cells priming. We were able to show that 10 times more Treg cells induce 100% survival of the skin graft, while the injection of lower ratios delayed the rejection.

First, we characterized the migration of Treg cells and effector T cells to the skin graft, since Treg cells were previously shown to infiltrate to and persist in the graft during the establishment of tolerance (156). Using our model, we demonstrated that both Treg and ABM cell populations in the skin graft express CCR4, suggesting a directed migration to the inflamed skin. Nevertheless, re-transplantation of short-term tolerated grafts on immunocompetent C57BL/6 mice did not result in graft tolerance and graft rejection was not correlated with the presence of Treg cells in the skin, since similar numbers were detected in the graft of the 2:1, 5:1, 10:1 and Treg groups. These data indicate that tolerance is established in the draining LNs rather than in the peripheral tissue (157, 158).

In order to develop and suppress allograft rejection Treg cells need to occupy the LNs (159). In agreement with this, high numbers of Treg cells were present in the dLNs of transplanted mice. Higher numbers of adoptively transferred Treg cells resulted in lower numbers of effector ABM cells in the LNs. By calculating the ratio of Treg to ABM cells in the LNs on day 9, we found that a ratio higher then 1 delayed rejection (5:1 group) while a ratio higher then 2 (10:1 group) correlates with long-term tolerance.

One mechanism by which Treg cells might impair the activation and proliferation of ABM cells is by influencing the activation state of DCs (160). Isolation of $CD11c^{high}$ DCs from the dLNs of transplanted mice with or without different T cells ratios (ABM, 10:1) did not induce activation, proliferation or cytokine release by ABM cells in vitro (own data). However, in vivo analysis showed that donor bm12-DCs were not impaired in migration to

the dLNs and in the expression of costimulatory molecules, indicating that Treg cells do not inhibit direct antigen presentation by inducing a tolerogenic phenotype.

Treg cells were previously shown to suppress T cell responses by inhibiting activation and proliferation of effector T cells in various animal models (161, 162). Our data reveal that although Treg cells do not alter the expression of activation molecules like CD25, CD44 and PD-1, they still impair the proliferation and the cytokine release of effector ABM cells. Higher Treg:ABM ratios in the LNs of bm12-transplanted mice prevented effector T cell function independent of DC activation.

Next, we wondered whether Treg survival is sustained in the LN and whether the Treg-mediated suppression might alter the LN environment.

Because of the recent discovery of TRCs in modulating IFN-γ mediated T cell responses (105), there is a lack of knowledge about TRCs cell biology. Therefore, we studied the function of TRCs during Treg mediated tolerance to MHC class II mismatched skin grafts. TRCs have the potential to inhibit the proliferation of CD8 T cells via nitric oxide (NO) release (104, 120) or PD-L1 surface expression (104, 120) in response to IFN-γ. In our assays we characterized the effect of IFN-γ/IFN-γR signaling on TRCs and found that they up-regulated transcription of NOS2, NOS3, IAb, PD-L1, ICAM-1 and VCAM-1. These findings are in agreement with published observations. In addition, we demonstrated that increased transcription correlated with increased surface expression of IAb, PD-L1 and ICAM-1 on TRCs. The higher transcription of ICAM-1, VCAM-1, IAb, IL-7, TSLP and PD-L1 on TRCs isolated from allograft-transplanted compared to syngraft-transplanted mice in C57BL/6 mice suggested a dependency on IFN-γ signaling. Indeed, the absence of IFN-γ signaling in IFN-$\gamma^{-/-}$ and IFN-$\gamma R^{-/-}$ mice (own data) abolished the differences in gene transcription seen during allo-responses, suggesting that IFN-γ is the main modulator of TRCs activity during acute alloresponses in immunocompetent mice. NOS transcripts were

not significantly different during acute alloresponses, suggesting that other mediators, including TNF and IL-1β, contributed to the release of nitric oxide (own data and (104, 120)).

To address whether T cells might influence the biological activity of LNSCs under steady state conditions we analyzed the transcriptional profile of TRCs in T cell deficient mice. The transcriptional profile of $Rag2^{-/-}$ and $CD3e^{-/-}$ TRCs was not different compared to C57BL/6 TRCs and the number of TRCs was similar in all strains, suggesting that the presence of T cells under non-inflammatory conditions does not induce expansion of TRCs and remodeling of the LN. The higher expression of IAb on the surface of C57BL/6 TRCs indicates an important role for T cells homeostasis (150). In summary, T cells have only a minor impact on TRCs activity under steady state conditions, while inflammatory stimuli and T cell activation can lead to modulation of TRCs activity.

Since Treg cells downregulate IFN-γ production by ABM cells, we hypothesized that Treg-mediated tolerance might influence the TRC compartment. Indeed, uncontrolled IFN-γ responses during viral infections have been shown to induce LNSCs damage (98, 112, 117, 163). However, Treg-mediated suppression of IFN-γ response did not change composition and proliferation of LNSC during alloresponses.

The major functions of LNSC are the release of chemokines that promote the recruitment of immune cells to the LN and the provision of survival factors for naïve and activated T cells. As several groups have described, CCL19 and CCL21 are the main chemokines to attract CCR7 expressing DCs and T cells to the LNs (164). CCR7 is not only important for recruitment, but also for the intranodal mobility inside the LNs (107, 108). Moreover, Treg cells require LN occupancy and CCR7 signaling for their activation and function (158). The expression of CCR7 on both ABM cells and Treg cells was similar in all groups, but Treg cells are required to maintain the transcription of CCL19 and CCL21 in TRCs. We

hypothesized that this contributes to Treg cell occupancy of the LNs and suppression of effector responses at the 10:1 ratio. However, lower numbers of transferred Treg cells were unable to block graft rejection by preventing down regulation of CCL19 and CCL21 in TRCs isolated from the 2:1 group. In this case, the resultant high numbers of Treg cells might therefore be a consequence of Treg expansion driven by IL-2 released from activated ABM cells.

Inflammatory cytokines up-regulate adhesion molecules on the surface of LECs, including ICAM-1 and VCAM-1 (95, 165-167). In the LNs, ICAM-1 and VCAM-1 are expressed on the surface of TRCs, HEVs and resident DCs (95, 97, 101, 168). Interestingly, ICAM-1 and VCAM-1 transcripts were increased in the TRCs isolated from tolerogenic 10:1 mice, but surface expression of ICAM-1 was only slightly increased and VCAM-1 was rather downregulated by the presence of Treg cells. The downregulation of VCAM-1 could be a consequence of Treg-mediated suppression (169) of IFN-γ release. The upregulation of ICAM-1 might be dependent on the presence of other cytokines, like TNFα. In fact, Treg cells were shown to inhibit Th1 responses by impairing IFN-γ but not TNFα production (161). Whether interaction of ICAM-1 and VCAM-1 on TRCs with LFA-1 and VLA-4 on Treg cells respectively might influence suppression was not addressed. Since upregulation of ICAM-1 is restricted to the 10:1 group, we can speculate that it might support the suppression of T cells since it was shown that the majority of Treg cells express LFA-1 in the LNs, which increases their suppressive capacity in vivo (170). In addition, the expression of ICAM-1 on TRCs might lead to the adhesion of $CD11b^+$/$Mac\text{-}1^+$ DCs (171, 172), which can drive mature DCs to differentiate into regulatory DCs. The tolerogenic phenotype of DCs in vitro might be a consequence of their increased interaction with stromal cells. Changes in the interactions of DCs and T cells with TRCs in vivo might

influence their activity by the local upregulation of adhesion molecules and the release of cytokines.

TSLP was highly produced by TRCs in the 10:1 group, suggesting that Treg cells might need TSLP during Treg-mediated tolerance. In the periphery, TSLP is mainly described to be involved in the generation of Th2 responses by modulating DCs (16-18, 58). The release of TSLP by human thymic epithelial cells activates a subpopulation of DC to express CD80 and CD86, which may be critical for the differentiation of auto-reactive T cells into Treg cells (59). However, the role in the maintenance and function of Treg cells in the periphery remains unknown.

In addition to TSLP, the transcription of the analogue cytokine IL-7 was enhanced in the 10:1 group. We hypothesized that stronger adhesion of T cells to TRCs might facilitate T cell access to this survival factor. IL-7 was induced following the activation of allogenic T cells in transplanted C57BL/6 mice and by TNFα alone or in combination with IFN-γ in vitro (own data). Since Treg cells only partially suppress IFN-γ release (own data) and are unable to shutoff TNFα (161), TRCs might respond by induction of IL-7 transcription. The lower IL-7 transcripts levels in the ABM and 2:1 group on day 9 might be the result of a different kinetic of effector cytokine release. Hence, preliminary data suggests that despite the absence of T cells in the LN on day 3, IL-7 transcription in the ABM group was induced in TRCs. In the future we plan to examine IL-7 production in the 10:1 group by transplanting IL-7-hCD25 mice, which express the human CD25 protein under the IL-7 promoter (117, 173). This mouse would allow us to detected even small amounts of produced IL-7 by staining the hCD25 protein.

Treg cells in the 10:1 group downregulate not only the IFN-γ, but also the IL-2 production of ABM cells in vitro. In addition, Treg cells were shown to downregulate the IL-2 mRNA on responder CD4 T cells after IL-2 signaling (174). The fact that IL-2 production by T cells in

the periphery is transient and Ag-dependent (175) might explain the low numbers of both ABM and Treg cells in the LN of syngrafted Rag2$^{-/-}$ mice. Accordingly, high Treg numbers were recovered in the 2:1 but not in the 10:1 group of allotransplanted mice underscoring the IL-2 dependence of Treg cells in homeostasis and activation (84). New data provide evidence that IL-7 can help Treg cell homeostasis in the periphery in the absence of IL-2 (83). In our transplantation model, the increased transcription of IL-7 by TRCs might improve Treg survival and maintenance of graft tolerance.

IL-7R is downregulated after activation on conventional T cells, as a consequence of the IL-2 released from activated T cells (176), and is reexpressed on memory T cells (54, 177). Lower expression of IL-7R on Treg cells isolated from the 10:1 and Treg group on day 6 indicates signaling after release of IL-7 from TRCs rather than IL-2 from ABM cells.

Although our results do not rule out the possibility of an additional mechanism for IL-7R down-regulation, IL-7 recognition by Treg cells leads to down-regulation of IL-7R and STAT5 phosphorylation in naïve Treg cells. Since, CD3-activation of Treg cells in the absence of exogenous IL-2 also showed down-regulation of IL-7R, it is possible that IL-7 signaling can still occur. Treg cells survived better in the presence of IL-7 in vitro and exhibited higher levels of the IL-2Rα chain CD25, similar to the 10:1 situation in vivo. In addition, suppression assays in vitro showed that Treg cells were enhancing their suppressive capacity on ABM proliferation. These data suggest that IL-7 signaling might influence the ability of Treg cells to bind IL-2 produced by activated T cells. Increased surface expression of CD25 in Treg cells would result in sensitization to low concentration of IL-2 by better capturing free IL-2, as shown for activated CD25$^{+}$ T cells (178). However, IL-2Rα alone binds IL-2 with low affinity without signal transduction and by presenting it to the IL-2Rβ and $\gamma_c$ forms a high affinity IL-2R complex (179). It would be interesting to

investigate whether IL-7 also induces the up-regulation of IL-2Rβ and $\gamma_c$ chains resulting in a high affinity binding of IL-2 and signal transduction. As a consequence, Treg cells could increase sequestration of IL-2 produced by effector T cells. Thus, Treg suppression of ABM proliferation in the presence of IL-7 was increased in vitro.

Activation of Treg cells in the presence of IL-2 and TCR signaling can lead to expansion of a population of highly suppressive short lived cells expressing TIM-3 and often PD-1 (90). IL-7 stimulation during Treg activation in vitro did not induce and modulate the IL-2-dependent TIM-3 up-regulation. These findings suggest that the higher surface expression of TIM-3 on Treg cells from the 2:1 and 10:1 groups might be a consequence of IL-2 signaling. Whether the high level of IL-7 in the 10:1 group increases the survival of short lived Treg cells is currently under investigation. However the over stimulation or blocking of the IL-7 pathway in vivo was not influencing the rejection or the Treg-mediated tolerance establishment.

Taken together, our data show that Treg cells suppress the activation and IFN-γ release by effector T cells and modulate the TRCs to release IL-7, which might be important for suppressing skin graft alloresponses by maintaining high numbers of Treg cells in the LNs. Moreover IL-7 sensitizes and might facilitate the recognition of IL-2 to induce expansion better and suppression of effector T cells.

# 6 Acknowledgments

First of all I would like to thank my supervisor Simona Rossi for giving me the opportunity to work in her lab and especially for choosing me as the first PhD student of her career. It was a great experience to build up a lab together! I would like to thank you for the support, the personal and scientific esteem you demonstrate towards me. I also highly appreciated during the years the opportunity you gave me to grow my scientific independence, and to directly contribute to build up the project. All together, these experiences will be fundamental to give me the personal and scientific strengths to start the new adventure I will be facing as a Post doc.

I would like to thank my doctor father Ed Palmer. It was a pleasure to be in close contact with your lab and be able to participate to your lab-meeting where you were always giving us valuable suggestions. I thank you also for our “evening discussions” about science and scientific career…really like a doctor father should do!

I thank Daniela Finke for her scientific contribution to the project, provided through discussions and ideas during committee-meetings and to have accepted to be my co-referee.

And now a really special thank to Mathias! In these years he was a colleague, mentor and friend that helped me with constant encouragement, positivity and competence. Our passion for research always generated vivid scientific discussions in the lab and at the Cargo Bar. These were always a great opportunity for me to learn how to think scientifically and how to defend own ideas. Your support was always fundamental and I am very happy to have lived and shared this experience with you!

I thank the people working in the animal facility and sorting facility of our institute who did an excellent job, which was fundamental for the realization of my experiments.

I thank my two English-speaking colleagues and friends Carolyn and Sarah for correcting the final version of my thesis. Your help was really precious!

I deeply thank all the special friends that were supporting me throughout these years, that were always ready to help and understand me in the good and bad times and that were rendering this experience unique.

Last but not least, I would like to thank my family who always believed in me and gave me the strength to go on!

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# 8 Curriculum Vitae

**Maria Anna Sofia Broggi**

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**Personal Information**

| | |
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| Date of birth: | 04. February 1986 |
| Place of birth: | Milan, Italy |
| Citizenships: | Swiss and Italian |

**Professional Aim**

Highly motivated researcher seeking for new scientific challenges as Post-Doc Scientist in an enthusiastic, dynamic and goal-oriented environment.

**Professional Experience**

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| 2009 – present | **PhD student** in the Lab of Prof. Simona Rossi, Immunoregulation Lab, Department of Biomedicine, University Hospital Basel, **Basel, Switzerland**<br>I investigate the role of regulatory T cells and their possible interaction with lymph node stromal cells in a mouse model of skin allograft transplantation.<br>Supervisor: Prof. Simona Rossi, Doctor father: Prof. Ed Palmer, Referee: Prof. Daniela Finke |
| 2007 – 2009 | **Master student** in the Lab of Prof. Gennaro De Libero, Experimental Immunology Lab, Department of Biomedicine, University Hospital Basel, **Basel, Switzerland**<br>Title of Master Thesis: “Characterization of invariant natural killer T cells in atherosclerotic patients” (Grade: 5.5/6)<br>Supervisor: Prof. Gennaro De Libero, Referee: Prof. Jean Pieters |
| 2006 – 2007 | **Bachelor student** in the Lab of Prof. Paola Castagnoli and Prof. Francesca Granucci, Immunology Lab, Department of Biotechnology and Bioscience, University of Milano-Bicocca, **Milan, Italy**<br>Title Bachelor Thesis: "Study of the role of interferon type 1 and of interleukin 18 in the activation process of natural killer cells by dendritic cells" (Grade: 110/110)<br>Supervisor: Prof. Francesca Granucci |
| 2013 – present | **Italian teacher and translator**, CVB International, **Lausen** (BS), **Switzerland**<br>Individual italian lessons and translation of documents from german and english to Italian upon request. |
| 2009 – 2010 | **Tutor**, Biozentrum, University of Basel, **Basel, Switzerland**<br>Lectures were hold for an entire semester and where addressed to first year biology students. The goal of the course was to discuss scientific questions to gain a common basic knowledge to start successfully the biology bachelor studies. |
| 2005 – 2006<br>2002 – 2004 | **Educational Assistant** in after-school classes and ski-camps for elementary and secondary school students, Swiss School of Milan, **Milan, Italy** |

**Educational Background**

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|---|---|
| 2009 – present | **PhD in immunology** and cell biology, Department of Biomedicine, University Hospital Basel, **Basel, Switzerland** |
| 2007 – 2009 | **Master in Molecular Biology**, Department of Biomedicine, University Hospital Basel, **Basel, Switzerland** |
| 2004 – 2007 | **Bachelor in Biotechnology**, University of Milano-Bicocca, **Milan, Italy** |
| 2000 – 2004 | **High school with Eidgenössische Matur** (MAR), Swiss School of Milan, **Milan, Italy** |

**Technical Skills**

- **Cellular biology and immunology techniques**: isolation, cultivation and stimulation of primary cells from murine secondary lymphoid organs and human blood; Ag-specific and non-specific T cell activation and suppression assays; transwell activation assays; MLR; blocking experiments with antibodies in vivo and in vitro; generation of Ag-presenting cells; ELISA, ELISpot; FACS (up to 10 colors intra- and extracellular), first insights in RNA silencing technologies and basics in confocal microscopy.
- **Mice in vivo experiments**: anesthesia; intra peritoneal, intra venous, intra dermal and subcutaneous injections; adoptive transfer of T cells; skin transplantation, immunization of mice with proteins/peptides and different adjuvants including CpG and LPS.
- **Molecular biology and biochemical techniques**: RNA extraction, cDNA synthesis, quantitative and real time PCR, DNA and protein electrophoresis, Western blot analysis.
- **Computer skills**: Microsoft Office Package, Mac Office Package, GraphPad Prism, FlowJo, Summit, ChemDraw, Papers.

**Congress Participations and Publications**

Selected oral presentations:

- *"Does the LN environment sustain allo-tolerance in mice?"*
  **Immunomeeting**, Department of Biomedicine, University Hospital Basel (2011, **Basel, Switzerland**)
- *"Treg cells and tolerance: the only players?"*
  **V. World Immunoregulation Meeting** (WIRM) on "Innate and Adaptive Immune Response and Role of Tissues in Immune Regulation" (2011, **Davos, Switzerland**)
  **XXIII. Meeting of Swiss Immunology Ph.D. Students** (2011, **Wolfsberg, Switzerland**)
- *"Transplantation tolerance in mice: T cells and more"*
  **Immunomeeting**, Department of Biomedicine, University Hospital Basel (2010, **Basel, Switzerland**)

Poster presentations**:**

- *"Treg cells modulate chemokines and cytokines in lymph node stromal cells"*
  **American Association of Immunology** (AAI) Meeting (2012, **Boston, USA**)
- *"Treg cells and tolerance: the only players"*
  **Summer School in Advanced Immunology**, ENII (2010, Capo Caccia, **Alghero, Italy**)
  **XXII. Meeting of the Swiss Immunology Ph.D. Students** (2010, **Wolfsberg, Switzerland**)

Publications**:**

- Broggi M. AS., Schmaler M., Page N., Palmer E., Rossi S.*, The role of lymph node stroma cells in allograft tolerance* (Manuscript in preparation)

Additional congress participations:

- **Neurex Workshop on „Multiple Sclerosis“** (2009, Department of Biomedicine, University Hospital **Basel, Switzerland**)
- **Second Basel Immunology Focus Symposium** – Immunological Tolerance: How to make it / How to break it (2009, Department of Biomedicine, University Hospital **Basel, Switzerland**)

**Awards**

- Selected participant for the **Novartis WIN** (Woman into industry) **Programm** (2012, mentoring program of Novartis in collaboration with the University of Basel, **Basel, Switzerland**)

  During this one-year program each mentee was supervised and coached by an experienced specialist or manager (mentor) from Novartis and had the unique possibility to enter in contact with many experts of the different fields of Novartis in order to discover new career paths and establish a valuable network.
- Selected participant for **Global Perspective Program, Preparing future academic leaders** (2012, Program of the International affairs of the University of **Basel** in collaboration with the Virginia Tech University, **Virgina, USA).**

  PhD students from different disciplines from both Universities got together to exchange ideas on how to improve and create a global higher education system by comparing the present swiss and US system. The program ended with an oral presentation of our ideas at the Swiss Embassy in **Washington**, **USA**.

**Courses**

- **Basics in project management** (2011, Didactic education for academic employees, University of **Basel, Switzerland**)
- **Successful acquisition of money for research grants** (2011, Didactic education for academic employees, University of **Basel, Switzerland**)
- **Career planning for PhD and Postdoc** (2011, Didactic education for academic employees, University of **Basel, Switzerland**)
- **Didactic and Rhetoric courses for academic education** (2011-present, Didactic education for academic employees, University of **Basel, Switzerland**)
- **Introductory Course in Laboratory Animal Science**, LTK1 Modul 1 (2009, Institute of Laboratory Animal Science, University of Zurich Irchel, **Basel, Switzerland**)

**Languages**

**Italian** (native language), **german** (second native language), **english** (good in speaking and writing), **french** (intermediate knowledge)

**Special interests**

Play the piano, traveling, cooking, and skiing

**References**

**Prof. Simona Rossi**, simona.rossi@unibas.ch, 0041 61 3287773
**Prof. Gennaro De Libero**, gennaro.delibero@unibas.ch, 0041 61 2652327
**Prof. Ed Palmer**, ed.palmer@unibas.ch, 0041 61 2653120